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THE CARNEGIE INSTITUTION OF WASHINGTON
ALFRED G. MAYER, Director

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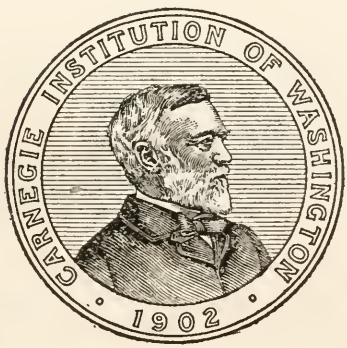
VOLUME XI



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CONTENTS.

PAGE.

I. *Nerve-Conduction in Cassiopea xamachana.* By Alfred Goldsborough Mayer. 15 figures 1–20
Methods and Corrections 3
Experiments 9
Summary 18

II. *The Hydrogen-ion Concentration, CO_2 Tension, and CO_2 Content of Sea-Water.* By J. F. McClendon, C. C. Gault, and S. Mulholland. 24 figures 21–69
Definitions and Abbreviations 23
Results applied to Oceanography and Measurement of Respiration-rate of Marine Organisms 27
Calibration of an Improved Indicator Method for use on Shipboard and in Poorly Equipped Laboratories 42
New Apparatus for the Electrometric Determination of Hydrogen-ion Concentration and for Gas Analysis 48
Historical Notes 65
Literature List 69

III. *Variability of Eggs and Sperm of Sea-urchins.* By A. J. Goldfarb 71–87
Technique 73
Concentration of the Germ-cells 74
Variation in Size and Shape of Eggs 77
Variations in Jelly Layer 78
Variation in Membrane Formation 79
Variation in Cleavage 80
Correlation of the Different Variations 84
Discussion 84
Conclusions 86
Bibliography 87

IV. *Analytical Search for Metals in Tortugas Marine Organisms.* By Alexander H. Phillips 89-93
Method 91

V. *On the Composition of Cassiopea xamachana and the Changes in it after Starvation.* By S. Hatai. 1 figure 95–109
Normal Growth of Different Parts of the Body 97
Analysis of Entire Body 99
Percentage of Water 99
Percentage of Nitrogen in Total Solids 99
Percentage of Ash in Solids 100
Analysis of Different Divisions of the Body 100
Percentage of Water 101
Percentage of Nitrogen in the Solids 101
Chemical Nature of the Gelatinous Substance of Cassiopea 102
Experiments on Starvation 103
Analysis of the Divisions of the Starved Cassiopea 108
Literature Cited 109

PAGE.

VI. *The Habits and Reactions of a Comatulid, Tropiometra carinata.* By Hubert Lyman Clark....111–119
Systematic Position.... 113
Habitat.... 114
Color and Size.... 114
Food.... 115
Locomotion.... 116
Reactions to Light.... 116
Reactions to Temperature.... 117
Reactions to Salinity.... 118
Reactions to Certain Chemicals.... 118
Summary.... 119

VII. *Studies on the Physiology of the Nervous System of Cassiopea xamachana.* By Lewis R. Cary. 18 text-figures....121–170
Technique of the Experiments and Some Results of Operations that apply equally to all Experiments.... 125
Experimental.... 130
Influence of the Sense-Organs on the Loss in Weight during Starvation.... 149
Influence of Sense-Organs on the Rate of Central Metabolism as measured by CO_2 Production.... 155
Influence of Sense-Organs on the Change in Rate of Pulsation in Response to Changes in Temperature.... 159
Relation between Area of Tissue Enervated by a Single Sense-Organ and rate of Pulsation.... 161
General Discussion.... 164
The Relation of Muscular Activity to the Rate of Metabolic Processes in Cassiopea.... 164
Summary.... 166
Literature Cited.... 169

VIII. *The Chemistry of Light-Production in Luminous Organisms.* By E. Newton Harvey. 1 text-figure....171–234
Studies on the Ostracod Crustacean *Cypridina hilgendorfii*.... 174
Studies on Fireflies.... 193
Studies on a Pennatulid, *Cavernularia haberi*.... 202
Studies on Luminous Bacteria.... 209
Studies on a Squid, *Watasenia scintillans*.... 222
Studies on a Fish, *Monocentris japonica*.... 223
Studies on Oxidative Chemiluminescent Reactions with Substances of Definite Composition.... 224
General Conclusions.... 233
Bibliography.... 234

IX. *A Physiological Study of Specific Gravity and of Luminescence in Noctiluca, with Special Reference to Anesthesia.* By Ethel Browne Harvey.....235–253
Light Response in Noctiluca.... 237
Change of Osmotic Pressure.... 238
Salts of Sea-Water.... 241
Acids and Alkalies.... 242
Potassium Cyanide.... 243
Absence of Oxygen.... 244
Temperature.... 244
Centrifuging.... 245
Electricity.... 245
Response of Injured Cells.... 246
Location of Luminous Material.... 246
Anesthetics.... 247
Summary.... 251
Literature List.... 253

PAGE.

X. *Polychætous Annelids from Florida, Porto Rico, Bermuda, and the Bahamas.* By Aaron L. Treadwell. 3 plates....255–268
Family Syllidæ.... 257
Family Polynolidæ.... 258
Family Phyllodocidæ.... 262
Family Leodicidæ.... 263
Family Sabellidæ.... 266
Literature.... 268

XI. *The Microscopic Structure of Striped Muscle of Limulus.* By H. E. Jordan. 3 plates....273–290
Material and Methods.... 276
Skeletal Muscle.... 276
Cardiac Muscle.... 279
Literature.... 282
Discussion.... 283
Summary.... 287
Bibliography.... 289
Description of Illustrations.... 290

XII. *Hemopoiesis in the Mongoose Embryo, with Special Reference to the Activity of the Endothelium, including that of the Yolk-Sac.* By H. E. Jordan. 5 plates....291–312
Material and Methods.... 294
Hemopoiesis in Yolk-Sac.... 294
Cell-Clusters of the Aorta.... 298
Endothelial Hemogenesis in Intraembryonic Mesenchyma.... 301
Cell-Clusters of the Pericardium.... 304
Discussion.... 304
Addendum.... 305
Bibliography.... 311
Description of Illustrations.... 312

XIII. *Embryonic History of the Germ-Cells of the Loggerhead Turtle (Caretta caretta).* By H. E. Jordan. 6 plates....313–344
Material and Methods.... 316
Descriptive.... 319
Literature.... 327
Discussion and Conclusions.... 333
Summary of Results.... 340
Bibliography.... 342
Description of Illustrations.... 344

XIV. *Atresia of the Esophagus in the Embryo of the Loggerhead Turtle (Caretta caretta): A Normal Developmental Condition.* By H. E. Jordan. 4 plates....345–360
Material.... 348
Descriptive.... 349
Discussion.... 354
Summary.... 358
Literature.... 360

I.

NERVE-CONDUCTION IN CASSIOPEA XAMACHANA.

By ALFRED GOLDSBOROUGH MAYER.

Fifteen figures.

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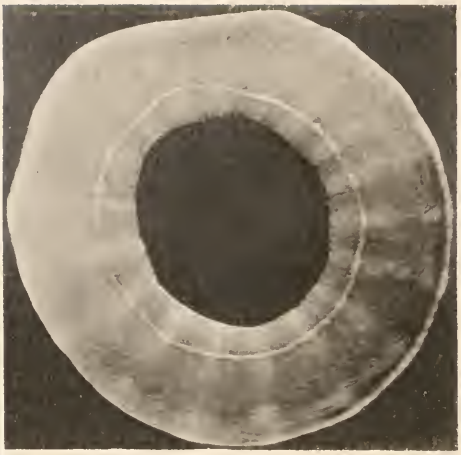

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1. Aboral view of *Cassiopea xamachana* from the moat of Fort Jefferson, Tortugas, Florida.
2. Oral view of subumbrella of *Cassiopea xamachana* with stomach and mouth-arms removed.
3. Ring of subumbrella tissue made by cutting off the marginal sense-organs and removing center of disk.

NERVE-CONDUCTION IN CASSIOPEA XAMACHANA.

By Alfred Goldsborough Mayer.

It is the author's privilege to acknowledge his indebtedness to kind friends for advice and aid: To Professor George A. Hulett, of Princeton University, for having had prepared in his laboratory the conductivity water used in this research; to Professor J. F. McClendon, of the University of Minnesota, for valued advice and aid, and to Professor L. R. Cary, of Princeton, for permission to make use of his recent determinations of the rate of nerve-conduction at different temperatures. By means of the generous interest of Professor E. G. Conklin and the authorities at Princeton I have enjoyed the excellent facilities afforded by the Biological Laboratory in Guyot Hall, wherein the kymograph records taken at Tortugas were studied and the results tabulated.

METHODS AND CORRECTIONS.

The object of this research was to obtain an accurate quantitative determination of the rate of nerve-conduction in natural and in diluted sea-water at constant temperature, and also to estimate the effects of various artificial sea-water solutions containing all or some of the sodium, magnesium, calcium, and potassium cations of sea-water. The effects of temperature upon nerve-conduction are also of great importance.

These studies were carried out in June and July 1916, upon *Cassiopea xamachana*, a rhizostomous scyphomedusa which is abundant in the salt-water moat surrounding Fort Jefferson at Tortugas, Florida, and is also common upon the bottoms of many of the shallow, semi-stagnant lagoons of the West Indian region. It is thus accustomed to a considerable range both in salinity and temperature, and being infested with commensal plant cells, it is in some measure independent of the oxygen-supply of the surrounding water, and even pulsates at a nearly normal rate in sea-water which has been deprived of air by boiling. The medusa thrives in confinement in glass aquaria and can be maintained alive in the laboratory for months while experiments are being performed upon it. Thus it is one of the most favorable of marine invertebrates upon which to conduct physiological studies.

An aboral view of *Cassiopea xamachana* is shown in figure 1, plate 1; figure 2 is an oral view of the subumbrella with the stomach and mouth-arms removed, and in figure 3 we see an annulus made by two circular cuts, one removing the marginal sense-organs and the other

cutting out the central stomach, and in addition a circular scratch cutting through the nervous and muscular layer of the subumbrella, thus separating the broad annulus of tissue into two rings. This subumbrella tissue becomes paralyzed through the removal of the marginal sense-organs, but the outer of the two annuli may then be stimulated by an induction shock until a contraction-wave going in *one* direction is entrapped in it, as illustrated in figure 4, and as has been described by Mayer, 1906, 1908.* Such a neurogenic contraction wave must travel continuously through the circuit of tissue which has entrapped it, and may maintain itself for days with but little change of rate, provided the temperature, CO_2, salinity, and hydrogen-ion concentration of the sea-water remain constant. Thus we have a means of entrapping a single neurogenic stimulus which remains practically uniform in intensity and rate for any desired length of time.

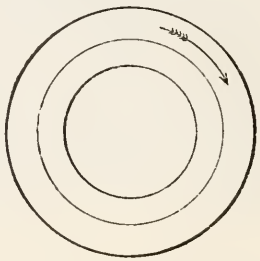


FIG. 4.—Diagram showing a single neurogenic pulsation-wave coursing in one direction through an annulus of subumbrella tissue.

Strong stimuli travel faster than weak ones, and thus if a wave stops it can not be started again at the same rate, for no two stimuli are received alike by the tissue. Such circuit waves may be stopped by counter-waves proceeding in the opposite direction against them, or blocked in the nerve net without apparent cause (fig. 5); or, if the tissue be exhausted, as in the absence of calcium, or by heat, cold, or CO_2, the wave may become irregular (fig. 13, lowest line) indicating that

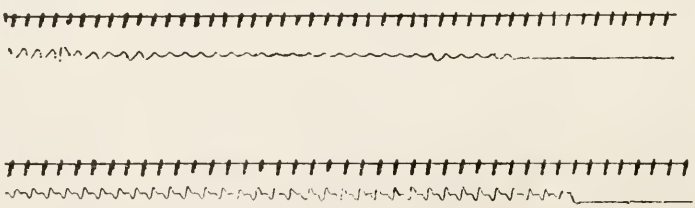


FIG. 5.—Two examples of sudden stopping of entrapped waves. Usually the wave becomes somewhat irregular before ceasing.

it is about to cease. In exhausted or weakened tissue, pulsus alternans may be displayed by the wave, the muscles becoming capable of responding fully only to every alternate (or even every second or third) return of the nerve stimulus (fig. 6, lowest line); but in normal, healthy tissues the wave is a full, regular sinusoid, the intervals being almost machine-like in their rhythmic sequence.

When a passive, paralyzed ring is activated by an induction shock, as in figure 7, muscular tonus is at once developed, and irregular and

*Carnegie Inst. Wash. Pub. No. 102, p. 116.

complex contraction waves proceed over it; but the larger waves travel faster than the smaller ones and thus overtake, and combine with them, in this manner forming a single component wave which appears upon the kymograph record as a smooth and regular sinusoidal curve.

All chemicals used in this research were Merck's "Reagent" in quality.

In taking these kymograph records a Jaquet chronoscope beating seconds, or a make-and-break pendulum, was used with each individual line, thus making the accuracy of the record independent of any changes in rate of the kymograph drum. One ascertained the number of seconds required for the contraction wave to travel 100 times around the ring in pure sea-water; and then the number of times it traveled around the ring in the same number of seconds in diluted sea-water gave its percentage rate in the diluted sea-water.

The average temperature of the surface water of the ocean at Tortugas during the day time in June and July is about 29° C., and the experiments of Harvey (1911), Mayer (1914), and Cary (1916), show that the rate of nerve-conduction augments in practically a right-line ratio

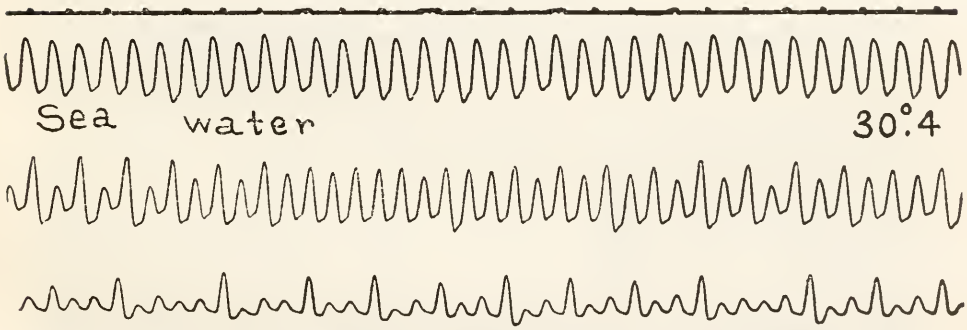


FIG. 6.—Pulsus alternans, indicating the weak, exhausted, or pathological character of the conducting tissue.

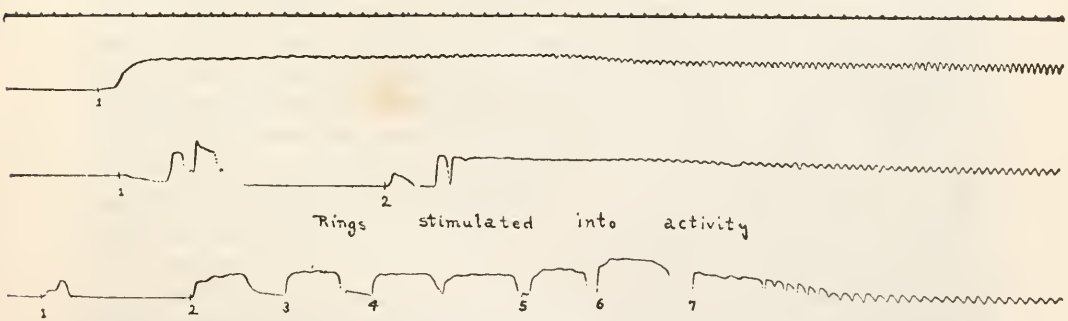


FIG. 7.—Three paralyzed rings being activated by induction shocks. A strong muscular tonus is at once developed, irregular waves proceed from the stimulated region, and finally a single regular sinusoidal wave going in *one* direction results from the interferences of the initial waves. The numbers represent successive attempts to stimulate each ring, the last one being successful.

from 17.5° to about 36° or 38° when it suddenly declines, the fall in rate indicating injurious effects, possibly due, as Harvey suggests, to the destruction of an accelerating enzyme. For several degrees, both above and below 29°, the rate changes about 4.5 per cent for each 1° C., and this correction was applied to all records. The records in diluted sea-water were usually taken at higher temperatures than those in the pure sea-water with which they were compared. The greatest average range was, however, not more than 1.79° C., but all records were reduced to the rate they would have exhibited had they been taken in a solution at the same temperature as that of the pure sea-water with which they were compared.

Another correction must be made on account of the osmotic interchange between the tissues of the ring and the diluted sea-water in which it is placed. Each experiment was made after the ring had been for one hour in 500 c.c. of fluid and a series of titrations with n/10 $AgNO_3$, using K_2CrO_4 as an indicator, showed the osmotic interchange had augmented the concentration of the diluted sea-water as follows:

TABLE 1.

Composition of the solution of 500 c.c. containing the pulsating ring.	Average percentage of concentration of the sea-water in which experiments were made.
Natural sea-water	100
95 vols. sea-water + 5 vols. distilled water	95.6
90 " " " + 10 " " "	90.56
80 " " " + 20 " " "	80.6
70 " " " + 30 " " "	70.8
60 " " " + 40 " " "	60.8
50 " " " + 50 " " "	50.8

The subumbrella tissue of *Cassiopea xamachana* is infested with commensal plant cells and thus when pulsating in diffuse daylight it gives out very little free CO_2. If placed in the dark, however, the sea-water surrounding the medusa soon becomes acid, due to the unreduced CO_2. All experiments were conducted during daylight hours in the diffuse light of the laboratory, and a series of tests showed that there was no appreciable change in the hydrogen-ion concentration of the pure or of the diluted sea-water after the pulsating ring had been in 500 c.c. of the solution for 1 hour. The laboratory was provided with a Leeds and Northrup potentiometer, which had been standardized by the U. S. Bureau of Standards; also with a Bovie potentiometer and two sets of standardized colorometric tubes made by Hynson, Westcott, and Dunning, one set being that of Rowntree, Levy, and Marriott, and filled with phenolsulphonephthalein, and the other set that of McClendon, wherein the tubes are filled with graded solutions of thymolsulphonephthalein. Both these sets of tubes were standardized by Pro-

fessor McClendon, who was engaged upon a study of the sea-water as a physiological fluid. He found that the P_H of the surface sea-water at Tortugas in summer, at about 29° C., ranges from 8.1 to 8.2, the range being due to variations in its carbonates. As is well known from the studies of L. J. Henderson (1913), these carbonates act as a buffer substance, and by ionizing they tend to neutralize the effects of any slight accession of acid to the water. Thus McClendon found that sea-water of P_H 8.1 diluted with an equal volume of distilled water of P_H 6.04 gave a solution of P_H 8.09. Also 50 per cent sea-water plus 50 per cent distilled water of P_H 8 gives a solution which is fully as alkaline as, if not slightly more so than, pure sea-water.

A number of tests of the change in hydrogen-ion concentration after pulsating rings had been in 500 c.c. of various dilutions of sea-water showed that in daylight there was no considerable change in 1 hour; while in four others it declined similarly in pure sea-water after 12 hours, and in 3 other rings placed for 12 hours each in 500 c.c. of 60 per cent sea-water plus 40 per cent of 8 P_H distilled water the P_H had declined to 7.95. The average change at the end of an hour was certainly not greater than from 8.1 to 8.0 P_H.

The thermometer used in this research was compared with one which had been recently standardized by the U. S. Bureau of Standards; both thermometers read to 0.1° C.

Ordinary distilled water often retains an acid reaction even after air freed from CO_2 by passing through tubes containing granulated soda-lime has been bubbled through it for 72 hours. Accordingly, Professor George A. Hulett, of Princeton University, very kindly offered to have prepared, under his direction and by his well-known method,* 144 liters of distilled water, which were sealed in 144 clean, steamed flasks of pyrex glass.† The hydrogen-ion concentration of the contents of each flask was tested separately and found to range from 0.80 to 1.0×10^{-6}, the average being 0.9×10^{-6}, or about 6.045 P_H.

This water was taken to Tortugas and used in all experiments herein mentioned. It retained a constant hydrogen-ion concentration and apparently no injurious elements were derived from the pyrex glass. In the series of experiments wherein the sea-water was diluted with distilled water of about 6 P_H, each pyrex glass flask was opened by breaking the narrow neck of the flask and the water was used as soon as possible, the opening in the neck of the flask being sealed in the intervals by a plug of soft paraffine. The distilled water of 6 P_H used in diluting the sea-water was not aerated and was thus deficient in oxygen. This, however, makes but little difference, for the commensal plant cells in the tissues of *Cassiopea* are so active in diffuse daylight that the medusa pulsates at a nearly normal rate even if placed in sea-

*1896; Zeitschift phys. Chemie, Bd. 21, p. 287.

†This water was prepared and tested by Mr. J. H. Yoe in Professor Hulett's laboratory.

water which had been boiled for an hour to expel its air, then restored to its former volume by air-free distilled water and cooled to normal temperature. Thus in daylight the medusa appears to be able, due to its plant cells, to supply nearly if not quite enough oxygen for its normal metabolism. The presence of free CO_2 in the surrounding sea-water is, however, very toxic and soon stops pulsation, and in darkness unreduced CO_2 accumulates, and thus the rate of movement is considerably slower than in daylight.

In another series of experiments, it was desired to dilute sea-water with distilled water of the same hydrogen-ion concentration as that of the sea-water itself. Accordingly, 50 flasks of Professor Hulett's distilled water were broken and the contents poured into a green-glass carboy which had previously contained Merck's distilled water. Air which had been freed from CO_2 by drawing it through glass tubes containing granulated soda-lime was then bubbled vigorously through this distilled water for 78 hours, after which the water had a P_H of 8. It was protected from the carbon dioxide of the atmosphere by permitting only air which had passed through soda-lime tubes to enter the carboy, and thus it retained an unchanged alkalinity remaining 8 P_H from July 4 to 11 inclusive, during which time 139 dilution experiments were conducted with it; after this its alkalinity began to decline while 26 additional experiments were made. The average for the entire series was 7.93 P_H or a hydrogen-ion concentration of 1.17×10^{-8}.

The fixity of the alkalinity at P_H 8 for so many days was probably due to OH ions derived from the alkaline green glass of the carboy, which may have been counterbalanced by a slight leakage of CO_2 through the rubber stopper of the carboy. Then, when the volume of the water became reduced, the surface over which solution of glass took place was relatively reduced in comparison with the surface capable of absorbing atmospheric CO_2, and thus the water finally tended toward acidity. Previous to its being used to hold the distilled water the carboy was cleaned by washing it with solutions of HCl, distilled water, KOH, absolute alcohol, and finally distilled water, taking precautions to prevent dust from entering.

The rings cut from the subumbrella of *Cassiopea* were attached to the kymograph lever by threads of catgut (fig. 15); while the rings themselves were each placed in 500 c.c. of solution contained in clean glass jars, covered between times of record-taking to prevent evaporation and to exclude dust.

Each ring was first placed in 500 c.c. of sea-water and its rate and temperature ascertained. It was then transferred to another glass jar containing 500 c.c. of a freshly made solution composed of 475 c.c. of sea-water and 25 c.c. of distilled water. At the end of an hour its rate was again ascertained on the kymograph and it was transferred

to another glass vessel which contained 90 per cent sea-water plus 10 per cent distilled water, and thus it was tested in more and more dilute solutions down to 50 per cent sea-water plus 50 per cent distilled water, after which it was replaced in natural sea-water. Rings which have been in 50 per cent sea-water recover more than 80 per cent of their normal rate within an hour after being replaced in pure sea-water, and, if replaced from 70 per cent sea-water, recovery is practically complete at the end of an hour. Even 50 per cent dilution produces, however, little or no permanent injury.

EXPERIMENTS.

Studies of recent years have shown the important influence of hydroxyl and hydrogen ions upon the rate of nerve-conduction. The free hydroxyl ion is a stimulant through a considerable range of concentration, but the free hydrogen ion is a depressant, excepting that in very weak concentration it acts as a moderate stimulant. Thus, if sea-water be diluted with acid distilled water of about P_H 5.5 (H-ion concentration 0.316×10^{-5}) the rate of nerve-conduction is augmented in slight dilutions, but it declines more and more rapidly in dilutions of 80 per cent sea-water plus 20 per cent distilled water and over. The muscular activity, as measured by the amplitude of the pulsation wave, usually declines steadily in sea-water diluted with acid distilled water of P_H 6, while when diluted with alkaline water of P_H 8 it maintains itself or even augments.

Table 2 shows the effect upon the muscular activity as measured by the amplitude of the pulsation wave in *Cassiopea* when taken from natural sea-water and gradually run into diluted sea-water composed of 50 volumes sea-water plus 50 volumes distilled water (50 per cent sea-water), at 30° C.

Table 2.

No. of experiments.	Hydrogen-ion concentration of the distilled water used in diluting the sea-water to 50 per cent.	Percentage of cases in which muscular activity was augmented by dilution of the sea-water.	Percentage in which muscular activity was decreased by the dilution of the sea-water.	Percentage unaffected by the dilution.
22	Acid distilled water of P_H about 6 at 30° C.	23	59	18
28	Alkaline distilled water of P_H 7.93 at 30° C...	57	25	18

Thus when sea-water is diluted with alkaline distilled water, more than half the rings show augmented muscular activity, whereas if the distilled water be slightly acid the muscles of more than half are depressed, and this depression is much more marked the greater the acidity of the distilled water used in diluting, so that with distilled water

of about 5.5 P_H the amplitude of muscular movement is hardly perceptible in 50 per cent sea-water. As the average temperature of the sea-water was about 30° C., the neutral point was about 6.86 P_H and thus it appears that even very slightly acid distilled water is usually a muscular depressant, whereas alkaline distilled water is a correspondingly efficient stimulant; this is the more remarkable when we consider that the hydrogen-ion concentration of the sea-water is very slightly augmented by dilution even with its own volume of distilled water of 6 P_H. Thus McClendon found that such a dilution of sea-water of 8.1 P_H changed the P_H of the solution to 8.09; in other words, the relative concentration of the hydrogen ions was increased only from 1 to 1.02, such is the efficiency of the buffer carbonates in preserving the normal alkalinity of the sea-water.

It will be recalled that Osterhout (1914)* finds that a slight concentration of HCl is at first stimulating to plants, but later (after the plasma membranes have been penetrated by the acid) it becomes depressant. It would seem that in *Cassiopea* a very slight relative increase of the H ion can not penetrate the cells, but acts only on the cell surfaces or membranes and in so doing becomes a stimulant; but in stronger concentration the cell membranes are penetrated and the H ion then exerts its well-known depressant effect.

The muscles are much more sensitive to changes of concentration in $H^{\cdot}$ or OH' ions than are the nerves.

The stimulating effect of a very slight increase of acidity or of alkalinity upon the rate of nerve-conduction is well shown if we place the pulsating ring in natural sea-water and then add about 5 per cent of acid distilled water of P_H about 5.5, for the rate suddenly augments about 5 per cent. Similarly, if we take the pulsating ring from normal sea-water and place it in sea-water which has been condensed by evaporation at normal temperature in the sun to about 90 per cent of its original volume, the rate augments on account of the high alkalinity of the evaporated sea-water.

Professor McClendon made up a series of acid and alkaline sea-waters. He added 23 c.c. of n/10 $NaHCO_3$ to each 1,000 c.c. of natural sea-water and after bubbling air through the solution for 12 hours the P_H was 8.26, and a film of calcium carbonate was precipitated upon the sides of the glass flask containing the solution. Similarly, he made acid sea-waters of P_H 7 by adding to each liter 24 c.c. of n/10 HCl and then aerating for 12 hours; and a sea-water of P_H 5.6 was obtained in the same manner by aerating for only half an hour.

The rates of nerve-conduction are nearly normal in all these solutions; but although the differences in rate are slight, they are such as we expected, the alkaline sea-water and the weakly acid sea-water are both slightly stimulating, while the more acid sea-water is depressant,

*Journ. Biol. Chemistry, vol. 19, p. 518.

as shown in table 3, the temperature of the solutions being 29° C. and the neutral point 6.88 P_H.

TABLE 3.

Character of the sea-water.	Average rate of nerve-conduction.	No. of observations.
Alkaline sea-water of P_H 8.26.....	102	6
Natural sea-water of P_H 8.15.....	100	....
Nearly neutral sea-water of P_H 7...	99.9	5
Slightly acid sea-water of P_H 6.5...	102	6
Acid sea-water of P_H 5.6.....	99.4	6

The effects are not so marked as those which are observed when the pulsating rings are taken from natural sea-water and placed in sea-water diluted with slightly acid distilled water, or in sea-water made alkaline by being concentrated by evaporation at ordinary temperatures exposed to the air; but they accord with expectation in so far as they go. Possibly the OH′ anion is stimulating even after it penetrates the cell membranes, while the hydrogen cation is depressant under these conditions.

The effects upon the rate of nerve-conduction in *Cassiopea* which resulted from diluting Tortugas sea-water with alkaline distilled water of P_H about 8 and with slightly acid distilled water of P_H about 6 are given in table 4, which shows that acid distilled water

TABLE 4. (Illustrated by figure 8.)

Composition of the solution.	Rate of nerve-conduction when sea-water is diluted with alkaline distilled water of P_H 7.93 at 29° C.	Rate of nerve-conduction when sea-water is diluted with slightly acid distilled water of P_H 6.04 at 29° C.
Natural sea-water of P_H 8.1 to 8.2.....	100	100
95 c.c. sea-water + 5 c.c. distilled water..	96.23	96.43
90 " " " + 10 " " "	91.44	94.38
80 " " " + 20 " " "	79.51	82.68
70 " " " + 30 " " "	73.91	71.95
60 " " " + 40 " " "	65.72	60.41
50 " " " + 50 " " "	54.16	50.83

is slightly stimulating in comparison with alkaline distilled water in weak dilution down to about 75 per cent sea-water plus 25 per cent distilled water, and then becomes relatively depressant in stronger dilutions. There are many examples of this in the author's past experiments published in 1914, 1915, and 1916, wherein the distilled water used for dilution was usually acid. An interesting parallel

appears to be that of acidosis in blood, wherein pathological effects appear when the amount of acid is so slight that the P_H is hardly affected, the buffer value of the carbonates and phosphates, however, being reduced.

It was due, indeed, to the acidity of the distilled water, and to my not realizing the marked effect of slight changes in temperature, that I was misled into entertaining the false idea that the curve of decline in rate in diluted sea-water resembles that of Freundlich's curve of adsorption, and that therefore adsorption may play a fundamental rôle in nerve-conduction. These recent experiments, wherein the sea-water

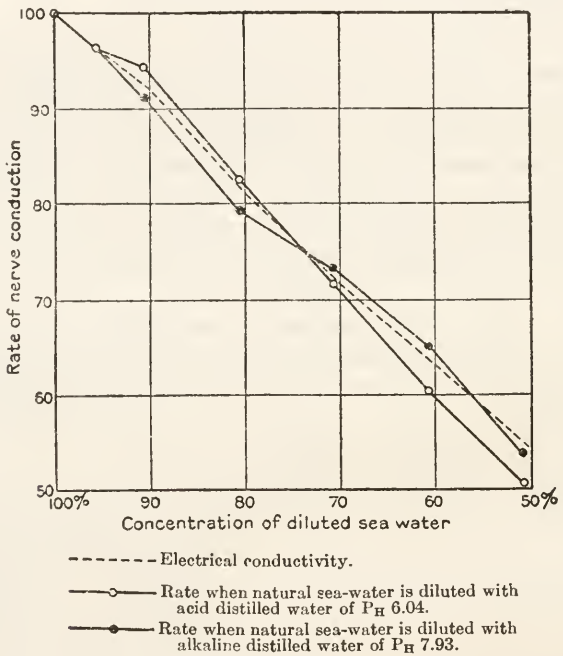


FIG. 8.—Showing decline in rate of nerve-conduction in *Cassiopea* in diluted sea-water, and corresponding decline in the electrical conductivity of sea-water. See tables 4 and 5.

was diluted with distilled water of P_H about 8, appear to show that adsorption has nothing to do with the matter.

Indeed, Ralph S. Lillie (1916)* has already called attention to the close resemblance between my curve for decline in rate of nerve-conduction and the corresponding decline in the electrical conductivity of the diluted sea-water, and in this latest and best-established curve of 1916, using distilled water of about P_H 8 to dilute the sea-water, the accordance with Lillie's expectation is almost perfect, as appears from table 5 and figure 8. It should be said, however, that the degree of ionization of the sodium, calcium, and potassium of the sea-water follows nearly the same law. Thus the rate of nerve-conduction, as

*American Journal of Physiology, vol. 41, pp. 126–136.

we shall show, appears to be directly proportional to the *concentration* of the surrounding cations of sodium, potassium, and calcium, magnesium taking an almost negative part in the control of rate of nerve-conduction in diluted sea-water.

Table 5 (illustrated by fig. 8) shows the rates of nerve-conduction in *Cassiopea* in Tortugas sea-water diluted with aerated, alkaline distilled water having a hydrogen-ion concentration of 1.17×10^{-8}, or 7.93 P_H.

TABLE 5.

Composition of the solution.	Average rate of nerve-conduction.	Electrical conductivity of Tortugas sea-water diluted with distilled water of P_H 7.8 at 30° C. determined by Kohlrausch's method.
Natural sea-water of P_H 8.1 to 8.2	100	100
95 c.c. of sea-water + 5 c.c. of distilled water	96.23	.....
90 " " " + 10 " " " "	91.44	92.16
80 " " " + 20 " " " "	79.51	81.38
70 " " " + 30 " " " "	73.91	71.53
60 " " " + 40 " " " "	65.72	64.26
50 " " " + 50 " " " "	54.16	54.08

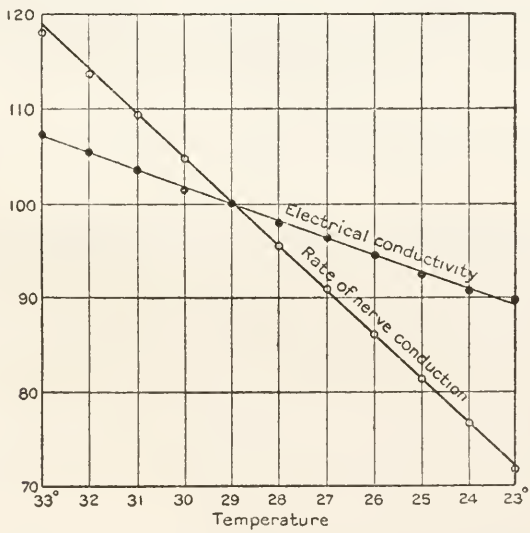


FIG. 9.—Showing that the temperature coefficient for the rate of nerve-conduction in *Cassiopea* is 2.5 times as great as that of the electrical conductivity of the sea-water.

The close resemblance between the decline in rate of nerve-conduction and the corresponding decline in electrical conductivity of the surrounding sea-water suggests but does not prove that the two phenomena are directly dependent one upon the other; but as we shall show later, this seems doubtful.

Lillie advocates an extension of the theory of Faraday and de la Rive that the transmission of the excitation state from the immediate site of activity to the adjoining resting areas is dependent upon an electrical local action of the same essential nature as that which is responsible for the etching or corrosion of non-homogeneous metallic surfaces, such as iron in contact with an electrolyte solution. Lillie calls this theory the "local action theory of conduction," and if, indeed, nerve-conduction be such a process, its rate must be proportional to the electrical conductivity of the conducting medium and the surrounding fluid (sea-water).

Lillie's hypothesis is, moreover, indirectly supported by the recent work of Adrian (1916),* who shows that after stimulation the recovery of conductivity is apparently complete at the same instant when the

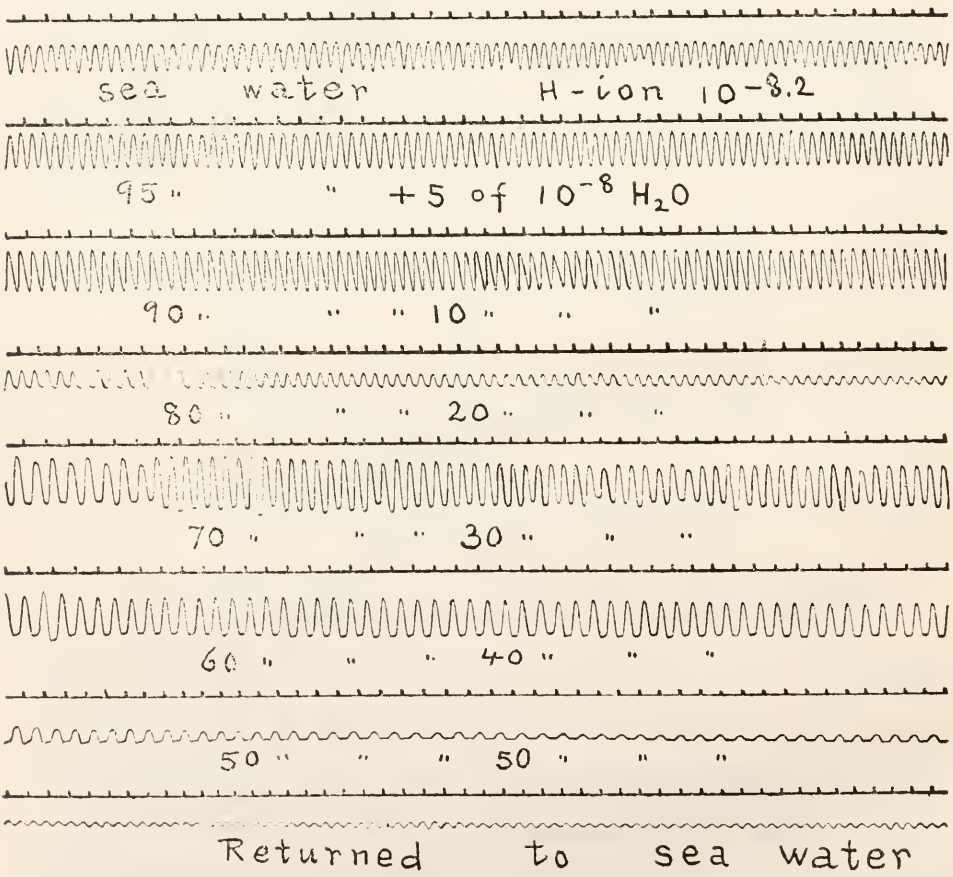


FIG. 10.—A ring of *Cassiopea* in diluted sea-water, showing decline in rate of nerve-conduction as dilution proceeds, followed by recovery of rate but not of amplitude upon being replaced in natural sea-water.

*Journal of Physiology, Cambridge, vol. 50, p. 345.

recovery of excitation is also completed and thus the two mechanisms may be identical and conduction may be merely the spread of a local exciting process.

Contrary to Lillie's hypothesis, we have direct evidence that the rate of nerve-conduction may be independent of the electrical conductivity of the electrolytic solution surrounding the nerves, for I have found (1915)* that if sea-water be diluted with 0.415 molecular $MgCl_2$ the rate of nerve-conduction is only slightly more depressed than if the sea-water be diluted with distilled water, or with dextrose; yet the $MgCl_2$ maintains a nearly normal electrical conductivity, while with distilled water or dextrose it declines in nearly the same ratio as the dilution. Nor do the experiments I have made with solutions containing some but not all the cations of sea-water support Lillie's view. Thus if the rate of nerve-conduction in 0.647 molecular NaCl be 55, it becomes 100 in 85.3 c.c. of 0.6 molecular NaCl+14.69 c.c. of 0.39 molecular $MgCl_2$. Here the electrical conductivity of the solution is somewhat reduced,

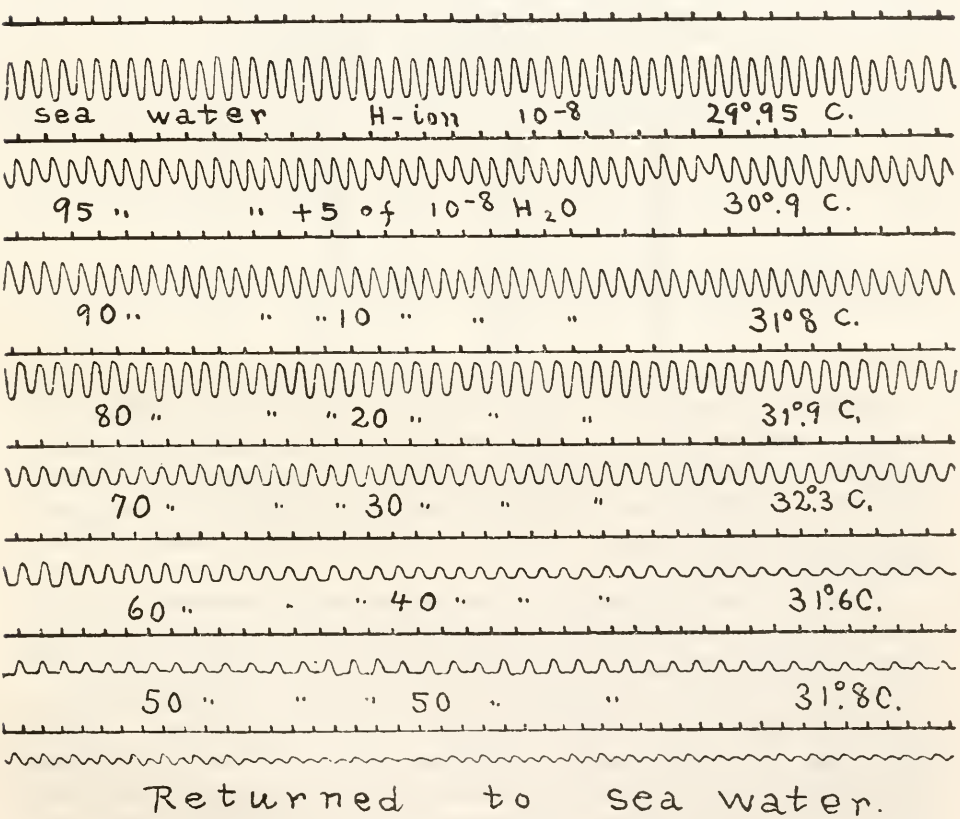


FIG. 11.—Showing changes in rate of nerve-conduction in a ring of *Cassiopea* in successive dilutions of sea-water, and the recovery of rate on being returned to natural sea-water from 50 per cent.

*Proceedings National Acad. Sciences, vol. 1, p. 270.

while the rate of nerve-conduction is much augmented. This is, of course, a striking instance of Loeb's law of the antagonism between a univalent and a bivalent cation, even though the bivalent cation in this case is magnesium, well known to be a depressant, especially for muscular activity in *Cassiopea.*

The temperature coefficient of nerve-conduction is 2.5 times as great as that of the electrical conductivity of the sea-water, as appears in table 6.

The rate of nerve-conduction in *Cassiopea* in heated sea-water was first determined by Harvey (1911) who found it to accelerate in a right-line ratio up to 35° to 38° C., at which point there was an abrupt decline in rate. These results were later confirmed by Mayer (1914), and Cary (1916). The average of the best experiments is shown in table 6.

TABLE 6. (Illustrated by figure 9.)

Temperature of the sea-water, °C.	Rate of nerve-conduction in *Cassiopea.* (Mean of determinations of Harvey, Mayer, and Cary.)	Electrical conductivity of Tortugas sea-water, determined by Kohlrausch's method.
23	71.3	88.9
24	76.3	90.7
25	81.17	92.6
26	85.8	94.4
27	90.74	96.2
28	95.47	98
29	100	100
30	104.47	101.6
31	109.2	103.5
32	113.4	105.3
33	117.8	107.1

The high temperature coefficient of the rate of nerve-conduction suggests that we may be dealing with a chemical reaction involving a compound composed of sodium, calcium, and some proteid element; the degree of ionization of which is considerably affected by temperature in the manner suggested by W. B. Hardy (1900), Quincke (1902), and Bayliss (1915).*

Possibly, also, the negative electrical potential associated with the wave of nerve-conduction may increase the surface tension of the alkaline colloidal particles, thus reducing their size, rendering them more soluble, and thereby increasing the concentration of the reacting ions.

In this connection, A. Mayer, A. Schaeffer, and E. Terroine (1907)† state that in a large number of alkaline organic colloids, the addition of a further negative charge, in the form of OH′ ions, caused a decrease in the size of the particles. Moreover, Hulett (1901)‡ has shown that

*Principles of General Physiology, p. 77.
†Compt. Rendus Acad. Sci., Paris, tome 145, p. 919.
‡Zeitschrift physik. Chemie, Bd. 37, p. 406.

the solubility of particles of barium sulphate is about inversely proportional to the size of the particles; and as colloidal particles are more or less soluble, one would expect their solubility to increase as the average diameter of the particles decreased.

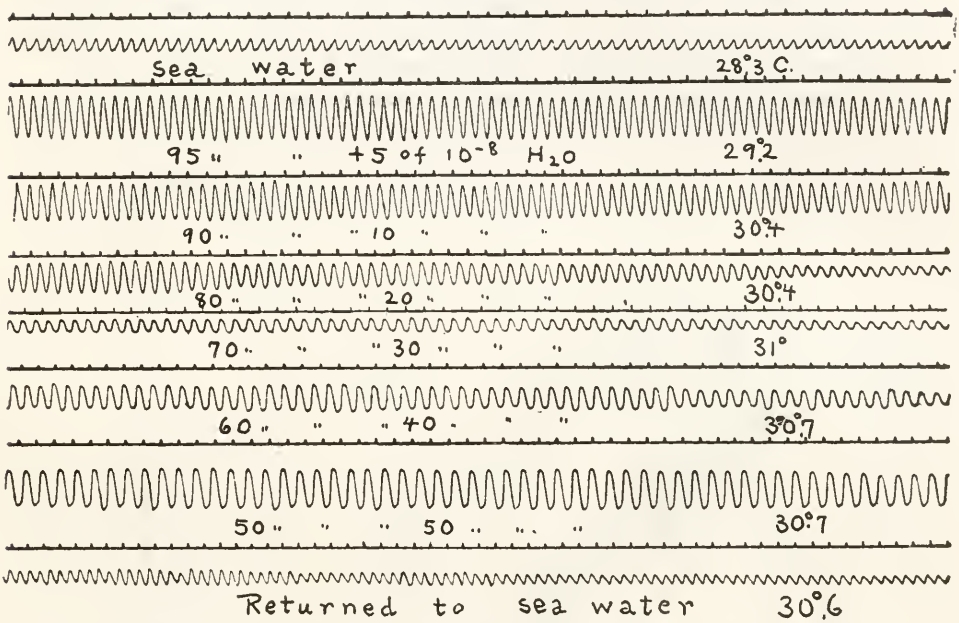


FIG. 12.—A ring of *Cassiopea* in diluted sea-water, showing decline in rate of nerve-conduction as dilution proceeds; also recovery of rate when the ring is replaced in natural sea-water.

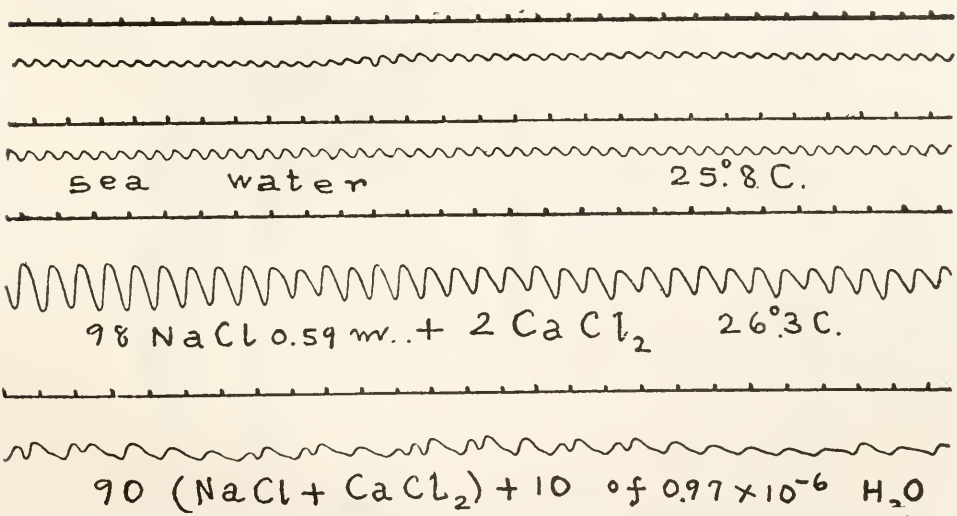


FIG. 13.—Showing change in rate of nerve-conduction in subumbrella ring of *Cassiopea* in NaCl+$CaCl_2$. In the lowest line the movement of the ring has become very irregular, indicating that it is about to stop.

SUMMARY.

Nerve-conduction is due to a chemical reaction involving the cations of sodium, calcium, and potassium. Magnesium is non-essential.

The probably high temperature coefficient of ionization of this ion proteid may account in some measure for the high temperature coefficient of the rate of nerve-conduction, which I find is 2.5 times as great as that of the electrical conductivity of the sea-water surrounding the nerve.

Our observations do not support the "local action" theory of R. S. Lillie (1916); for this maintains that the rate of nerve-conduction must

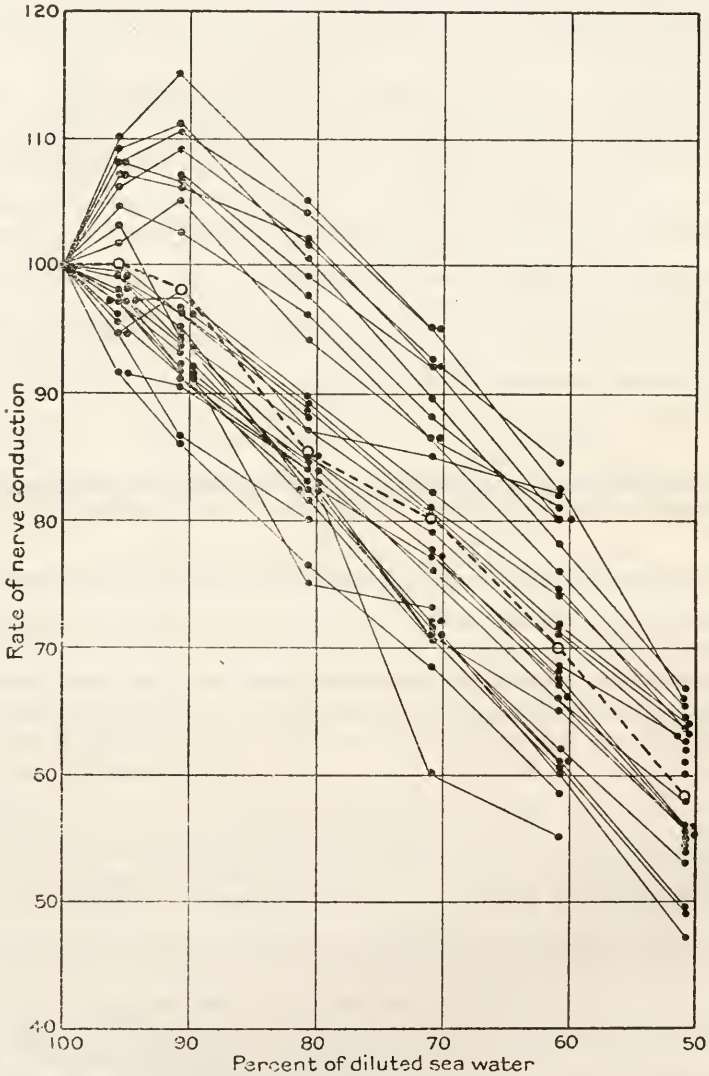


FIG. 14.—Illustrating table 7. Results of each individual experiment upon rate of nerve-conduction in *Cassiopea* in sea-water diluted with alkaline distilled water of hydrogen-ion concentration 1.17×10^{-8}. The heavy dotted line shows the observed average curve.

be a function of the electrical conductivity of the conducting tissue and of the electrolytic solution surrounding the nerve. It is found, however, that the rate of nerve-conduction is practically identical, whether we dilute sea-water with 0.415 molecular $MgCl_2$ or with distilled water—in other words, whether we maintain a practically constant electrical conductivity or reduce it in nearly the same ratio as the dilution.

Table 7, illustrated by figure 14, shows rates of nerve-conduction in the subumbrella tissue of *Cassiopea xamachana* in Tortugas sea-water of P_H 8.1 to 8.2 diluted with aerated distilled water of P_H about 8.0 at 29° C. The rate in pure sea-water at the same temperature as the diluted sea-water is assumed to be 100. The experiments were conducted at Tortugas, Florida, on July 4 to 11 and July 19 to 25, 1916.

TABLE 7.

[s.w. = sea-water. d.w. = distilled water.]

	Natural sea-water.	95 c.c. of s.w. + 5 c.c. of d.w.	90 c.c. of s.w. + 10 c.c. of d.w.	80 c.c. of s.w. + 20 c.c. of d.w.	70 c.c. of s.w. + 30 c.c. of d.w.	60 c.c. of s.w. + 40 c.c. of d.w.	50 c.c. of s.w. + 50 c.c. of d.w.
Average concentration of sea-water at time record was taken.....	100	95.6	90.56	80.6	70.8	60.8	50.8
Number of observations.	38	28	28	28	28	29	24
Observed average rate...	100	100.1	97.87	85.38	80.05	70.11	58.37
Probable error of observed average rate...	0	0.67	1.00	1.18	1.16	1.01	0.79
Temperature difference (°C.) between solution and pure sea-water in which rate was 100...	0°	0.86°	1.46°	1.53°	1.79°	1.51°	1.64°
Rate corrected for temperature by reducing observed rate 4.5 per cent for each 1° C. rise in temperature.....	100	96.23	91.44	79.51	73.91	65.72	54.16

Observed rates.

95 c.c. of s.w. + 5 c.c. of d.w.		90 c.c. of s.w. + 10 c.c. of d.w.		80 c.c. of s.w. + 20 c.c. of d.w.		70 c.c. of s.w. + 30 c.c. of d.w.		60 c.c. of s.w. + 40 c.c. of d.w.		50 c.c. of s.w. + 50 c.c. of d.w.	
91.5	99	86	96	75	87	60	80	55	70	47	62
91.5	99	86.5	96	76.5	88	68.5	80.5	58.5	71	49	62.5
94.5	99.5	90.5	96.5	80	88.5	70.5	81	60	71.5	49.5	63
94.5	100	91	97.5	81.5	89	71	82	60.2	71.7	53	63.2
95.5	101.5	91	98	82.5	89.7	71	85	61	74	54	63.5
96	103	91.5	102.5	82.5	94	71.5	86.5	61	74.5	54.5	64
97	104.5	92	105	82.5	96	72	86.5	62	76	55	64.5
97	106	92	106	83	97.5	72	88	65	78	55.5	65.5
97	107	93	106.5	83	99	73	89.5	66	80	55.5	66
97	107	93.5	107	84	100.4	76	92	66	80	56	66.7
97.5	108	93.5	109	84	101.5	77	92	67	81	56	....
98	108	94	110.7	84.5	102	77	92.5	67.5	82	58	....
98	109	94.5	111	85	104	77.5	95	68	82.5	60	....
98	110	95	115	85	105	79	95	68.5	84.5	61	....
Average 100.1		Average 97.87		Average 85.38		Average 80.05		Average 70.11		Average 58.37	

Table 8 shows the rates of nerve-conduction in subumbrella tissue of *Cassiopea xamachana* in Tortugas sea-water of P_H 8.1 to 8.2 diluted with non-aerated distilled water of P_H about 6 (H-ion concentration 0.9×10^{-6}) at 29° C. The rate in undiluted sea-water is taken to be 100. The experiments were conducted at Tortugas, Florida, in June and July 1916.

TABLE 8.

[s.w. = sea-water. d.w. = distilled water.]

	Natural sea-water.	95 c.c. of s.w. + 5 c.c. of d.w.	90 c.c. of s.w. + 10 c.c. of d.w.	80 c.c. of s.w. + 20 c.c. of d.w.	70 c.c. of s.w. + 30 c.c. of d.w.	60 c.c. of s.w. + 40 c.c. of d.w.	50 c.c. of s.w. + 50 c.c. of d.w.
Average concentration of sea-water at time record was taken.....	100	95.6	90.56	80.6	70.8	60.8	50.8
Number of observations.	51	20	27	25	28	26	22
Observed average rate...	100	99.1	97.91	87.96	77.28	64.33	53.79
Probable error of observed average rate...	0	0.74	0.88	1.16	1.13	0.59	0.90
Temperature difference (°C.) between solution and pure sea-water in which rate was 100...	0°	0.6°	0.8°	1.33°	1.54°	1.37°	1.23°
Rate corrected for temperature by reducing observed rate 4.5 per cent for each 1° C. rise in temperature.....	100	96.43	94.38	82.68	71.95	60.41	50.83

Observed rates.

95 c.c. of s.w. + 5 c.c. of d.w.		90 c.c. of s.w. + 10 c.c. of d.w.		80 c.c. of s.w. + 20 c.c. of d.w.		70 c.c. of s.w. + 30 c.c. of d.w.		60 c.c. of s.w. + 40 c.c. of d.w.		50 c.c. of s.w. + 50 c.c. of d.w.	
94	98	85	98	76.5	88	62.5	74.5	54	66.5	41	54
95	98	86	98.5	77	88	67	75	55.4	66.5	43.5	55
95.5	98	88	99	77.5	88	69.5	76.5	59.5	66.5	46.5	55.5
96	98	91	99.5	79.5	89	70	76.5	60	67	47	56.5
96.5	99	92	100	82	90.2	71	77.5	60	68	50	57.5
97	103	92	100.5	82	90.5	71	79	60.5	68	50	58.5
97	103	94	101	83	91	72.5	79.5	60.5	68.5	52	....
97.5	106	94	102	84.2	96	73	80	61	69.5	52.5	....
97.5	107	94.5	104	85	106	73	80.5	62	69.5	53	....
97.5	108.5	96	104	85	107	73.5	80.5	64	....	53	61
....	.....	96	105	85.5	109	74	82	64.5	70	53.5	62
....	.....	96	107.5	85.5	.....	74	83	64.5	72	53.5	63
....	.....	97	111	86.5	.....	74.5	91.5	64.7	....	....	65
....	.....	....	112	87	.....	....	96	65	....	....	....
....	99.1	....	.....	....	.....	....	107	66	....	....	....
Average 99.1		Average 97.91		Average 87.96		Average 77.28		Average 64.33		Average 53.79	

Fig. 15.—Taking a Kymograph Record from a Pulsating Ring. The chronoscope is not shown.

II.

THE HYDROGEN-ION CONCENTRATION, CO_2 TENSION, AND CO_2 CONTENT OF SEA-WATER.

By J. F. McCLENDON, C. C. GAULT, and S. MULHOLLAND.

Twenty-four figures.

CONTENTS.

PAGE.

Definitions and abbreviations 23
Results applied to oceanography and measurement of respiration-rate of marine organisms 27
Calibration of an improved indicator method for use on shipboard and in poorly equipped laboratories 42
New apparatus for the electrometric determination of hydrogen-ion concentration and for gas analysis 48
Historical notes 65
Literature list 69

THE HYDROGEN-ION CONCENTRATION, CO_2 TENSION, AND CO_2 CONTENT OF SEA-WATER.*

By J. F. McClendon, C. C. Gault, and S. Mulholland.

DEFINITIONS AND ABBREVIATIONS.

Since the volume of water changes with the temperature, the most accurate method of expressing the results of water analysis is to take the kilogram of water as the unit. The older method was to express results in grams per kilogram. In volumetric analysis it is more convenient to express results in grams per liter, and this method is equally accurate provided the water is brought to a standard temperaature, preferably 20°, for analysis, or the temperature is recorded in each case. Since the ratios of the weights of chemical elements or radicals that will enter into chemical composition with one another have been established with an accuracy more than adequate for oceanographic work, it is still more convenient to express results in gram-equivalents per liter.

A normal solution (abbreviated to n) is a solution containing in 1 liter the quantity of substance that will combine with 23 grams of sodium or 35.46 grams of chlorine. A mol-liter solution (abbreviated to m) is the molecular weight in grams (mol) of the substance contained in a liter of the solution, and may or may not be a normal solution, and where confusion is likely to arise, m and not n should be used. For example: if enough H_3BO_3 is added to a normal solution of NaOH to make a 2 m solution of the former, the result is a 0.5 m solution of $Na_2B_4O_7$. In the ordinary use of m we mean more correctly the *formal* concentration—*i. e.*, the molecular weight is taken from the ordinary chemical formula, regardless of the fact that several molecular species containing the substance in question may occur in the solution.

Where a particular molecular or ionic species is intended, it should be designated. Thus a normal solution of hydrogen ions contains 1.008 grams (or for practical purposes about 1 gram) of hydrogen ions per liter, regardless of the amount of hydrogen in other chemical states. It is not always possible to reduce such a quantity to a standard temperature, since some hydrogen ions may disappear or be formed during the change in temperature. This particular case is simplified by the fact that the normal concentration of H ions per liter is not changed very much by the ordinary changes in temperature, as

*This investigation was aided by a grant from the research fund of the graduate school of the University of Minnesota. We are indebted to Dr. H. A. Lubs and Professor L. G. Rowntree for some indicators.

will be explained later. It is more convenient to abbreviate the names of positive and negative ions thus: $H^{\cdot}$ = hydrogen ion, Cl' = chlorine ion.

Since concentrations per liter and per kilogram may be readily converted one into the other if the density of the solution is known, charts for computing the density of sea-water are given in figures 1

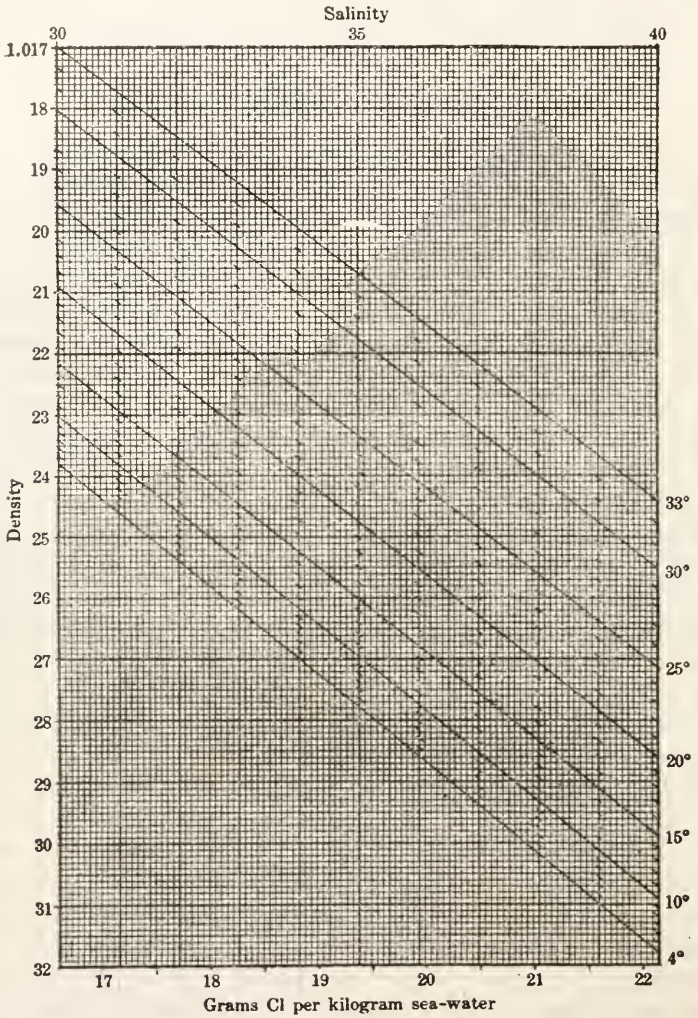


FIG. 1.—Conversion table for finding the density of sea-water (at any temperature), referred to distilled water at 4° from the salinity or the Cl in grams per kilogram. Each diagonal is for indicated temperature only.

and 2. This gives the actual density in question, as it is obviously insufficient merely to know the specific gravity at some standard temperature. The direct determination of the specific gravity by means of a floating hydrometer is often vitiated by large experimental errors, and many of these instruments are made for a standard temper-

ature on the obsolete Réaumur thermometer scale (14 R. = 17.5 C.), which is often unattainable aboard ship or at seaside laboratories. The best practice is to titrate the chlorides of the sea-water with silver-nitrate solution, using potassium chromate as indicator, and, if a constant temperature can not be maintained, to calibrate each new silver solution with standard sea-water. Since the temperature

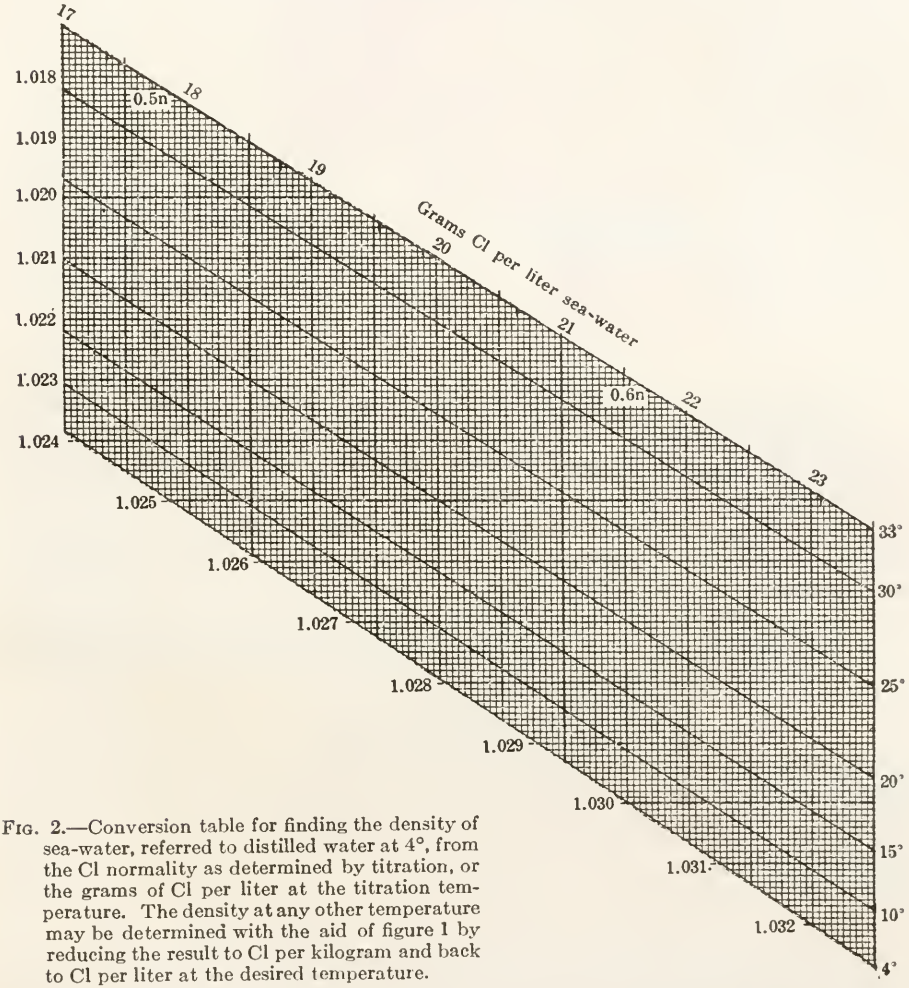


FIG. 2.—Conversion table for finding the density of sea-water, referred to distilled water at 4°, from the Cl normality as determined by titration, or the grams of Cl per liter at the titration temperature. The density at any other temperature may be determined with the aid of figure 1 by reducing the result to Cl per kilogram and back to Cl per liter at the desired temperature.

changes affect the volumes of the silver solution and sea-water about equally, they may be ignored. The concentration of sea-water was formerly expressed as the *salinity* or total salt-content, but it was found that the ratio of the chief salts in sea-water is remarkably constant, and the individual elements may be determined much more accurately than the total salts in one operation. Salinity is no longer determined directly, but is calculated from the chlorine titration according

to the Sörensen-Knudsen definition. If it is desired to know the salinity (S) more accurately than can be determined with the aid of figure 1, the following conversion table (Knudsen) may be used:

(Integral part and decimal point omitted in table.)

Cl per kilo 19.37 g = S 35.00										19.93 g = 36.00 S	
Cl	S	Cl	S	Cl	S	Cl	S	Cl	S	Cl	S
37	00	48	19	59	39	70	59	81	79	93	00
38	01	49	21	60	41	71	61	82	81	94	02
39	03	50	23	61	43	72	62	83	82	95	04
40	05	51	25	62	44	73	64	84	84	96	06
41	07	52	26	63	46	74	66	85	86	97	08
42	08	53	28	64	48	75	68	86	88	98	09
43	10	54	30	65	50	76	70	87	90	99	11
44	12	55	32	66	52	77	71	88	91	20	36
45	14	56	34	67	53	78	73	89	93	00	13
46	16	57	35	68	55	79	75	90	95		
47	17	58	37	69	57	80	77	91	97	20.00 = 36.13	
								92	99		

As already mentioned, the unit of the hydrogen-ion concentration is 1 n $H^{\cdot}$, or about 1 gram of hydrogen ions per liter. The quantity actually found in sea-water is about 1 gram in 100,000,000 liters, or 0.00000001 n. In order to avoid the use of so much space, this number is usually expressed as 10^{-8}, which is nothing more than a minus logarithm to the base 10—in other words, a common logarithm with the sign changed. We might write it thus: $-\log H^{\cdot} = 8$, but Sörensen still further abbreviated it thus: $P_H = 8$. It is necessary only to remember that when the hydrogen-ion concentration decreases the P_H increases in the following manner:

0.1 n $H^{\cdot} = P_H 1$ 0.00000001 n $H^{\cdot} = P_H 8$ 10^{-14} n $H^{\cdot} = P_H 14$.

The H-ion concentration of aqueous solutions is determined by the dissociation of water as affected by the temperature and the presence of acids or bases. There are about 55 mols of H_2O in a liter of water, that is, its concentration is 55 m in respect to the formula. Of these 55 mols, only about 10^{-7} m is dissociated into $H^{\cdot}$ and OH' if the water is pure. In other words, the concentration of $H^{\cdot} \times OH'$ is 10^{-14}. If we add acid we increase the H ions and if we add alkali we increase the OH ions, but the concentration of $H^{\cdot} \times OH'$ remains constant ($=10^{-14}$). This dissociation constant of water is abbreviated K_w, and hence $-\log K_w = 14$. Rise in temperature increases the dissociation of water; hence $-\log K_w$ decreases, being 14.07 at 20° and 13.73 at 30°, or a fall of 0.34; hence the P_H falls 0.17. We might expect the P_H of sea-water to fall the same amount as that of pure water with rise in temperature, but such is not the case. Rise in temperature causes increased hydrolysis of bicarbonates, and hence increases the OH-ion

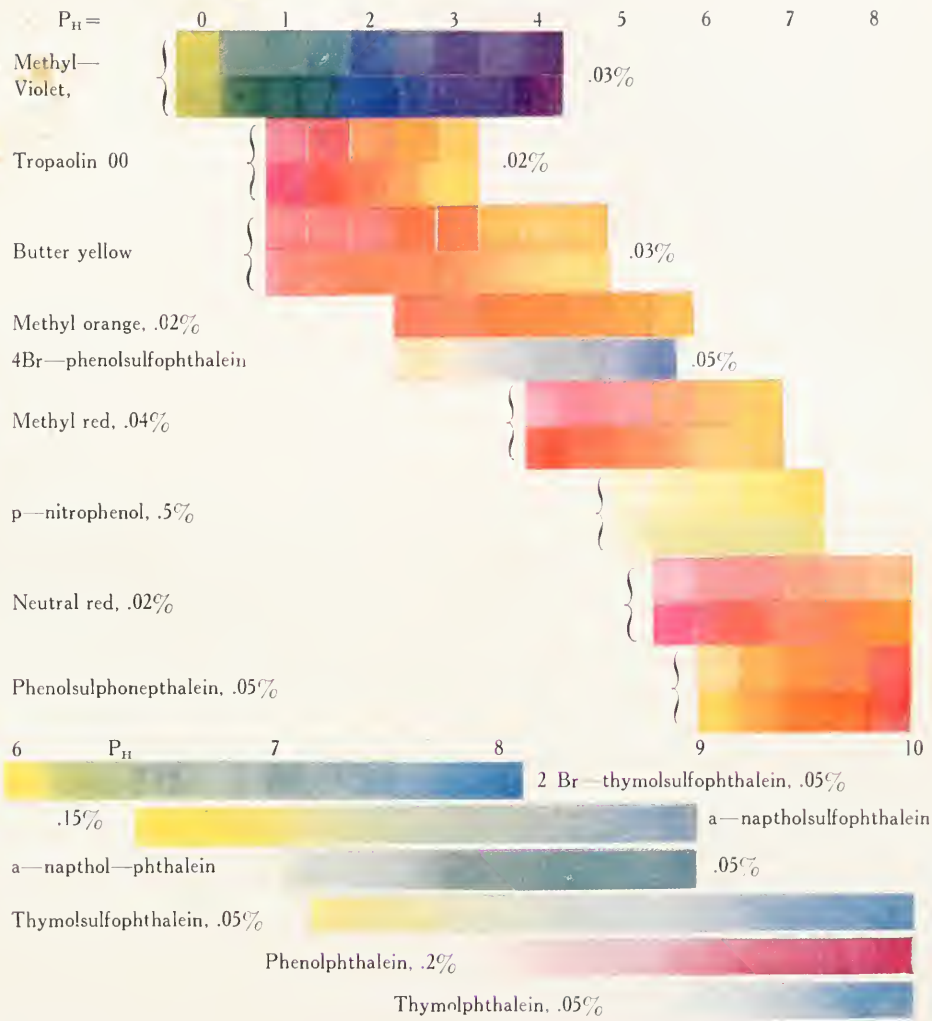


FIG. 3.—Conversion table for roughly determining the P_H of any aqueous solution. The solution is placed in a test-tube of 1 cm. bore together with 0.1 c.c. of the indicator solution of the indicated concentration and the color is compared with the chart while looking downward into the tube. In many cases two attempts to match the color were made, the continuous scale being made with the aid of water colors and the discontinuous scale with the aid of aniline dyes. About 0.3 must be subtracted from the P_H of sea-water as determined by this chart if sulfophthalein indicators are used, to correct for the salt error. For finer work the tubes described in this paper are recommended.

concentration, and these OH ions combine with the newly formed H ions to form water again. Any change in the H-ion concentration of sea-water due solely to change in temperature (*i. e.*, provided the temperature change is not allowed to cause a loss or gain of CO_2) is within the limits of error of our measurements. All measurements were made with the hydrogen electrode, which under proper conditions is affected only by the hydrogen ions. The indicators that were calibrated are weak acids and hence should be affected by H ions and not by OH ions. The indicators are affected by Na ions to a slight extent, as given in the calibration table. Temperature changes affect these indicators, but the ordinary changes in temperature affect them so slightly that it has not yet been possible to estimate the temperature correction. Furthermore, the temperature coefficient in sea-water and standard borax solution is probably the same.

The CO_2 tension of a solution is the fugacity or escaping tendency of CO_2. It is measured by estimating the partial pressure of CO_2 in an atmosphere in equilibrium with the solution—*i. e.*, the pressure of CO_2 required to prevent the escape of CO_2 from the solution. The air is not quite in equilibrium with the surface-water of the sea, so that it is not sufficient to determine the CO_2 partial pressure in the air unless it is shaken with sea-water. Since the average CO_2 in air is 3 parts in 10,000, the CO_2 pressure is given in parts per 10,000 of a standard atmosphere of 760 mm. Hg. The total CO_2 content of sea-water is the total amount of CO_2 that may be obtained from it by boiling or evacuating or aeration with indifferent gas after adding an excess of acid to decompose the carbonates. It is not in the form of a gas in sea-water, but exists in several molecular species: CO_2, H_2CO_3, HCO_3', CO_3'', and carbonates and bicarbonates of all of the bases present. The CO_2 is estimated as cubic centimeters of the dry gas at 0° and 760 mm., and may be reduced to milligrams by multiplying by 1.965.

The $H^{\cdot}$ concentration of sea-water is maintained fairly constant by the presence of salts of weak acids called buffers. These are, first CO_2 and second H_3BO_3, H_3PO_4, SiO_2, and As_2O_3, which are not lost by volatilization under conditions in the sea, and are collectively abbreviated to non-volatile buffers. The P_H of sea-water remains constant so long as these buffers (especially CO_2) are not increased or diminished in proportion with the bases combined with them.

RESULTS APPLIED TO OCEANOGRAPHY AND MEASUREMENT OF RESPIRATION-RATE OF MARINE ORGANISMS.

All of the P_H determinations were made with the hydrogen electrode, but at the same time the indicator method was calibrated so that the results may be duplicated by this more convenient method. The indicators used were mostly selected from the list shown in figure 3. It was found that the sulfophthalein series gave the most reliable

results because of the brilliancy of the colors and the fact that they are differently colored in acid solutions and hence the change is qualitative and not quantitative. An error due to a difference in concentration of the indicator may be at once observed, whereas this is not possible with phenolphthalein. Dibrom-o-cresolsulfophthalein has the same range as p-nitrophenol; dibromthymolsulfophthalein has the same range as phenolsulfophthalein; dibromthymolsulfophthalein has about the same range as a-naptholsulfophthalein.

Part of the work was done at Tortugas, Florida, and the sea-water was drawn directly from the sea into the apparatus, but owing to the inability to work at any temperature except 30° and also to the incompleteness of the library, the work could not be finished there, at least in one summer. Part of the results obtained at Tortugas have already been published (McClendon, 1916).

The work was brought to the present state of completeness in Minneapolis, and the experiments at 30° are in close agreement with those made at Tortugas. The sea-water that had been used in aquaria, even those of pyrex glass, was deficient in non-volatile buffer, due to the action of organisms, and could not be used in standard experiments. In all of the Minneapolis experiments one of the sulfophthalein indicators was added to the extent of 1 mg. to 100 c.c. of sea-water, and so increased the non-volatile buffer to this slight extent; this causes a small theoretical error, but was necessary in order to make possible the duplication of the experiments by the indicator method. There was not sufficient time for another series of experiments without the indicator, as the sea is too complex a solution to be treated in any simple mathematical manner, and it was necessary to make a large number of direct determinations over a wide range of conditions. Some of the determinations were repeated from 2 to 10 times before we were confident of the correctness of the results. During the investigation the solution of o-cresolsulfophthalein was accidentally lost and a new one was prepared in the same manner and from a bottle of the same label by the same manufacturer. It was found that a buffer mixture containing this indicator had the same color as a buffer mixture of 0.15 higher P_H colored with the old solution. This led to a repetition of much of the work. It has long been known that such an experience may be had with any indicator, but it is hoped that these excellent indicators may be manufactured in larger quantities, so as to insure greater uniformity, and that each yield be numbered and so labeled.

Alkaline solutions of borax and other buffer mixtures of standard P_H are affected by absorption of CO_2 by the air and solution of glass. Those sealed in 1 cm. nonsol glass tubes were found to keep for about a year. In order to increase their life and accuracy, new solutions of higher buffer value were standardized and sealed (by fusing the glass) in 24 mm. nonsol tubes containing the indicator. These tubes should

be good for 5 years, provided they are kept in the dark most of the time.[1]

Details of the indicator method and the colorimeter that we used are given on pages 44–46, and we will now pass on to the question of the duplication of our results by the indicator method.

We found the P_H of sea-water to be practically independent of salinity or temperature between 20° and 30°, and all of our experiments

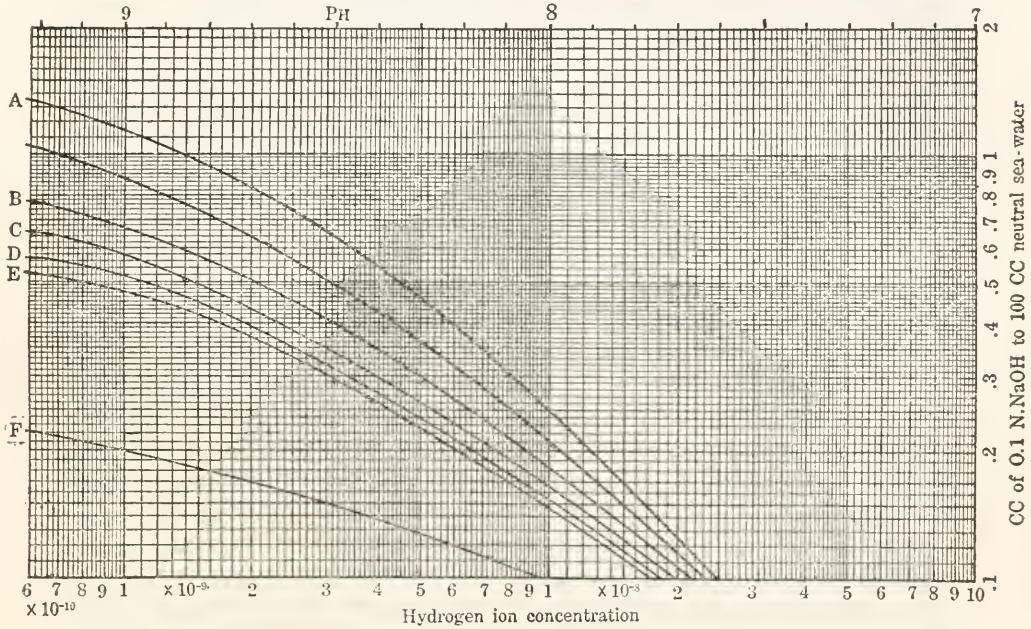


FIG. 4.—Results of electrometric titration of CO_2-free sea-water plus indicator with CO_2-free NaOH solution. A very slight excess of HCl is added to the water and the CO_2 driven off by boiling, the water is brought about to the neutral point ($P_H = 7$), and then titrated. On the ordinate is measured the logarithm of the number of cubic centimeters of 0.1 normal NaOH added to 100 c.c. of the neutral, CO_2-free sea-water, and on the abscissa is measured the P_H. At the bottom is a scale for conversion of the P_H into normality of H-ion concentration. The titrations were made on sea-water from the following sources: *A*, artificial sea-water containing 0.0015 molecular boric acid. *B*, sea-water from Tortugas, Florida, *C*, artificial sea-water containing 0.0008 molecular boric acid. *D*, Tortugas sea-water in which diatoms and other Protista had been grown in a pyrex flask for about 6 months. *E*, sea-water collected somewhere off Hatteras and kept in the Princeton marine aquarium about 6 years, the evaporation being compensated by addition of rain-water. *F*, artificial sea-water without any non-volatile buffer. Later experiments showed these data to be merely relative.

at 10° indicate that even this drop in temperature does not appreciably affect the P_H. Our values given for 0° are extrapolated from CO_2 data on the assumption that the P_H is independent of the temperature.

We found the P_H of sea-water to be determined solely by ratio of the concentration of buffers, including CO_2 and other weak acids, to the concentration of bases combined with them (excess base over strong acid).

[1]Duplicates of our tubes and colorimeter may be obtained from the manufacturers of the indicators.

Attempts were made at Tortugas and continued at Minneapolis to estimate the non-volatile buffers separately, and to find some reliable data in the literature, but this work has not reached a state for publication. It was found that phosphates and silicates are soluble enough, but it seems impossible to find enough silicate and phosphate in sea-water to account for all of the non-volatile buffers, even though we regard the results of analyses as being rough estimates. Borates were found in Tortugas sea-water and in the Princeton Marine Aquarium and in the Pacific Ocean by the senior author. An attempt was made to increase the non-volatile buffer by the addition of phosphates, but marine animals behaved abnormally in this water. The same was attempted with silicates, and plants grew luxuriantly in the artificial sea-water. No more accurate data were found when the Tortugas paper was sent to press (the paper was received for publication September 14), but about 6 weeks later the paper of Henderson and Cohn appeared, in which it was stated that artificial sea-water containing boron equivalent to 0.0015 boric acid had the same buffer value as sea-water. This led us to make a series of electrometric titrations, continuing those begun at Tortugas, on a series of natural and artificial sea-waters under CO_2-free conditions. The results are given in figure 4, and it was estimated from this and tonometer experiments that Tortugas sea-water has a non-volatile buffer value much less than 0.001 m boric acid, whereas by the growth of diatoms and other marine Protista in a pyrex flask the same water was reduced to less than this; and Princeton aquarium water, kindly sent by Dr. L. R. Cary, had a still lower non-volatile buffer value. It is not possible to determine the exact boric-acid equivalent from figure 4, and in estimating that of Tortugas water the P_H of artificial sea-waters and Tortugas sea-water at the same CO_2 tension were found to be about equal.

We have not been able to obtain a sample of sea-water with as high non-volatile buffer value as artificial sea-water of 0.0015 m boric-acid content. Veatch reported evidence that the shore-water off the southern California coast is in communication with unknown borax deposits. Dr. William E. Ritter kindly sent us a sample of this water, and it was found to give the same qualitative test for boric acid as any of the other sea-waters examined. It had a non-volatile buffer value equivalent to Tortugas sea-water. We feel justified, therefore, in the assumption that the non-volatile buffer value of sea-water of fairly normal salinity is a more or less constant quantity and is markedly changed only when organisms are kept a long time in a relatively small quantity of sea-water. The water at the surface of the ocean, where most of the organisms live, is constantly being renewed by vertical ocean-currents.

On the assumption that the weak acids in the sea have a somewhat comparable buffer value, the error that might arise in calculating the

CO_2 content from the P_H could be large only in case variation in non-volatile buffer is large. But CO_2 is more than 100 times as strong an acid as boric acid, the dissociation constant of the former being about $10^{-6.5}$ and of the latter $10^{-9.25}$. CO_2 may neutralize 4 times as much NaOH as boric acid is able to, the formulæ being Na_2CO_3 and $Na_2B_4O_7$ (although the solution is not neutral in either case, being alkaline, due to hydrolysis). We may assume, therefore, that the maximum error in estimating total CO_2 from P_H, due to variation in the non-volatile buffer, is negligible (not including any other sources of error).

The estimation of the excess base is as follows: Owing to the presence of boric and traces of phosphoric acid, phenolphthalein can not be used, since we have found that it gives a result about 5 to 10 per cent too low. Methyl red is not as sensitive and may give too high a result, and it is better to use the same indicator we used if comparative results are desired. 100 c.c. of sea-water are placed in an Erlenmeyer flask of resistance glass with etched mark at 100 c.c. and enough dibrom-o-cresolsulfophthalein solution to color it a distinct purple; then enough 0.01 n HCl is run in from a burette to turn it yellow. The flask, together with a resistance-glass or quartz beaker of distilled water, is boiled, preferably on an electric hot plate (alcohol flame is better than gas if electricity is not available). The sea-water will become purple again, and more acid should be added to turn it yellow and the boiling continued. Wooden tongs or a wooden model of a nut-cracker are preferable to a wire test-tube holder for handling the hot flask. The end-point is reached when just enough acid has been added to turn it yellow, and it does not turn purple on further boiling for 5 minutes, while the volume is kept approximately constant by additions of the boiling distilled water. Distilled water that has remained a long time in soft glass should not be used, and if water free from solid residue can not be obtained, it is better not to restore the volume, but to repeat the titration with the addition of all but 1 c.c. of acid before boiling and thus accelerate the evolution of CO_2. Prolonged boiling after the end-point has been reached is to be avoided, owing to the slight solubility of even the best resistance glass. It is theoretically possible to boil off traces of HCl after the end-point has been reached, but this process is necessarily extremely slow at so low an acidity. Normal sea-water will require a little more or less than 25 c.c. of 0.01 m HCl per 100 c.c. or 25 c.c. of 0.1 n HCl per liter, and this number (for example 25) is used to denote the excess base over strong acid (*i. e.*, the base combined with the buffer acids).

In titrating the non-volatile buffers the same procedure minus the indicator is followed (though it is possible to cut all of the quantities in half if necessary). The entire quantity of acid is added at first and the sea-water boiled about 15 minutes gently, in order to remove all of

the CO_2. Any CO_2 in the water will be estimated as non-volatile buffer and influence the result. The equivalent of about 1 c.c. of 0.1 n NaOH is added to the boiling sea-water; the volume is quickly and accurately restored with boiling distilled water, and the flask tightly closed with a rubber stopper. The NaOH solution must be free from CO_2 and contain little silicate. The method of its preparation and protection is given on page 45. The exact quantity of NaOH to be added can not be stated, but there should not be sufficient to cause precipitation of earthy hydroxides. It is best to have two series of tubes of known P_H, one colored with thymolsulfophthalein and the other with either o-cresolsulfophthalein or a-naphtholsulfophthalein—in other words, a total range of $P_H = 7.5$ to 9 or greater, but only the first series of tubes are necessary. Two nonsol test-tubes of exactly the same bore as the standard tubes and with etched marks at the volume to be titrated are provided, each with a rubber stopper with a central perforation closed with a glass rod. The required quantity of indicator is placed in each of the tubes and sea-water from the flask poured in (to the mark) and the stopper quickly inserted. Care must be taken never to breathe toward the test-tube unless it is tightly closed. The tube with thymolsulfophthalein is first examined and compared with the standard tubes and its P_H recorded. The glass rod is removed from time to time and small quantities of 0.1 n HCl are run in from the burette. The tube is shaken and its P_H is recorded, together with the burette reading.

This process is repeated until the limit of the indicator is nearly reached, when enough indicator is added to correct for the increased volume and any error due to dilution of the indicator is recorded. The same process is repeated with the other indicator and the results are compared with figure 4.

If the excess base is known the total CO_2 may be determined from the P_H by using the conversion table in figure 5. The three diagonal lines are for sea-waters containing 23, 24, and 25 excess base respectively. The smallest possible difference in CO_2 per liter that we have been able to determine by means of the standard tubes is 0.1 c.c., but in order to do this it is necessary to have a finer gradation of tubes than are ordinarily used. Such a degree of accuracy is only relative, since the absolute accuracy of the conversion table probably does not exceed 1 per cent. The diagonals in the conversion table are drawn as straight lines, but this is merely an approximation. If they were extended they would have to be curved or lose in accuracy. The sea is of too complex a composition to admit of any simple mathematical relations. Empirical formulæ, such as the one developed by Fox for estimating the total CO_2, may be discovered, but they are only approximations. Exact data may be obtained only by direct experiment, and the result depends on the technique.

If the sea-water contains more than normal non-volatile buffer, the CO_2 content will be less than that read from the table, and *vice versa*, but the quantitative side of this relation has not been thoroughly investigated. We may picture it in the following manner: Suppose the water is sealed in an air-tight container so that CO_2 can not escape. If we add boric acid part of it will remain free and increase the number of H ions; but since it is so weak an acid, this change will be slight. Part of the boric acid will displace CO_2 from bicarbonates and some of the CO_2 will dissociate and increase the number of H ions still more. Since boric acid is weaker than CO_2 it will not decrease the P_H as much as the same number of molecules of CO_2; hence the error in estimating the CO_2 will not be as great as the same molecular concentration of boric acid. SiO_2 would have even less effect than boric acid, but phosphoric acid would have a relatively greater effect. We know of no determinations of As_2O_3 in sea-water, but Bang found 4 mg. per kilogram of fish.

The conversion table in figure 5 has a very limited range, and the further it is extended graphically the greater the error. We did this for the Princeton aquarium water because it had the very abnormal excess base of about 44.5. We determined the total CO_2 and P_H on a portion of it and Dr. L. R. Cary determined the P_H of another portion and the change in P_H on further additions of CO_2. The results are:

Total CO_2 from experiment.	Total CO_2 from table.	Difference.	P_H
88.4	82.2	− 6.2	8.1
99.4	100.0	+ 0.6	7.5
114.8	115.2	+ 0.4	7.0
143.7	127.5	−16.2	6.6

The difference of 6.2 c.c. CO_2 at P_H 8.1 is only 0.0004 m, but the difference at P_H 6.6 is probably partly due to an error in the extended table or the experimental data. It may be remarked that the history of this abnormal water does not completely solve the question of the origin of the difference in excess base. It is possible that some concrete may have been dissolved, although it was said to have been paraffined. Concrete may possibly have been dissolved by the rain-water used to compensate for evaporation, or limestone dust may have gotten into the rain-water by being blown onto the roofs. These characteristics of the water were not investigated when it was first received.

In using the conversion table in figure 5 for the study of the respiration of marine organisms, only relative values are necessary, and it is thought wholly adequate for the purpose. It would be of little advantage to extend the table to a greater P_H range unless it is first absolutely established that the abnormal P_H does not make the organisms physiologically abnormal and that oxygen is still present in the water (see

oxygen scale at left of figure 5 for temperate and tropical seas). The scale at the extreme left shows that the temperature is raised only 0.04° by animal respiration that uses up all of the oxygen in the warmer seas. This shows the impossibility of the existence of a warm-blooded animal with gill respiration.

The oxygen-content of sea-water may be estimated from the P_H, since the differences between cubic centimeters of CO_2 and O_2 in 2 samples of water of the same excess base are reciprocal.

The change in P_H with change in CO_2 tension is affected only by the temperature, as shown in conversion table in figure 6.

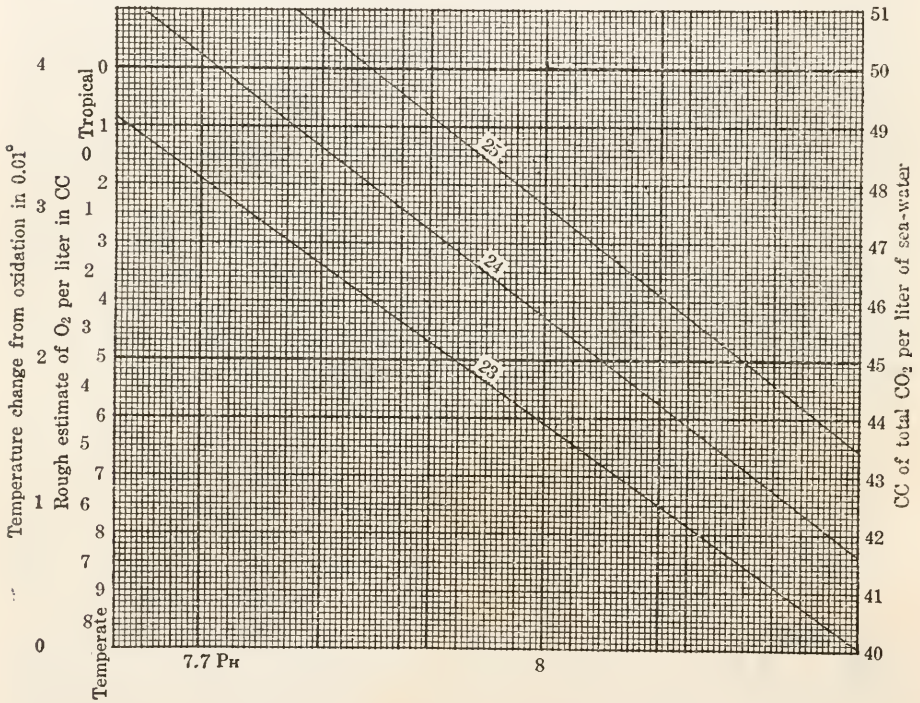


FIG. 5.—Conversion table for determining the total CO_2 and O_2, and the rise in temperature of sea-water due to the respiration of marine animals, from the excess base and P_H. The three diagonals are for three values of the excess base. On the abscissa is given the P_H and on the ordinate are four scales, one for the total CO_2, one for the approximate O_2 of tropical oceans, one for the O_2 of temperate oceans of about 20°, and one for the approximate rise in temperature due to respiration of marine animals, in hundredths of a degree. This estimate of rise in temperature is a little too small, but can not be calculated exactly.

Changes in salinity theoretically affect the CO_2 tension and hence CO_2 content of sea-water. Fox divides the CO_2 into two parts, one of which obeys Henry's law and never exceeds 1 per cent of the total. It is this small fraction that is affected by salinity, but the changes in salinity ordinarily met with in the larger oceans change this fraction only about 1.5 per cent, and the total change is exceedingly small. We have not been able to determine any effect of salinity on P_H or CO_2 content.

Henderson and Cohn state that if the CO_2 tension is maintained constant (at about 0.0004 atmosphere) the P_H changes from 8.03 at 30.73 salinity to 8.1 at 34.6 S. We were unable to confirm this result with the hydrogen electrode. Henderson and Cohn used the indicator method of Sörensen and Palitzsch, and the difference between successive standard tubes as recorded in several of the papers of these authors is $P_H = 0.1$. The tubes were exposed to the air, but were occasionally renewed. Their results were sometimes expressed in figures of four decimal places, but this probably was not intended to have more than mathematical significance. In fact, we found that, with the most accurately measured sealed tubes placed in a colorimeter that brings the centers of the tubes together on a sharp line, we could not distinguish with certainty any difference smaller than about P_H 0.02, even in the most sensitive region of the range of the most brilliant indicators. The fact that Henderson and Cohn observed P_H 8.06 at S 31.58, 8.07 at 32.05, and 8.06 at 32.45, indicates that they made small errors in the estimation of P_H or CO_2 tension or temperature. Sörensen and Palitzsch give the salt error of their indicators at 20 and at 35 salinity, and Henderson and Cohn may possibly have attempted to estimate the change in salt error from S 30.7 to 34.6, but they do not mention the fact.

We have recalibrated our salt errors about 50 times, but the different determinations do not coincide within an error of less than 0.025 P_H. On the average it was found to be necessary to subtract 0.05 from the observed indicator P_H (sulfophthalein series) when the salinity was increased from 30 to 37.7 in order to obtain the same result as with the hydrogen electrode. If this correction is applicable to Henderson and Cohn's indicator for this range (probably phenolphthalein) the increase in P_H with rise in salinity from 31.58 to 34.6 is reduced to 0.2 and is about the limit of the smallest possible difference that can be determined with the eye, whereas the range of salinity is as great as is ordinarily met with in the larger oceans. We feel safe, therefore, in assuming that the influence of salinity on the relation of CO_2 tension to P_H at any specified temperature in negligible. In fact, Henderson and Cohn do not state the variation in temperature. In our experiments the temperature was observed within 0.05° and controlled within 0.2° during the majority of the experiments and to within 0.1° during critical periods.

In figure 6, the curves for 30°, 20°, and 10° are taken from the means of many determinations, whereas the curve for 0° was extrapolated. The results of Henderson and Cohn's experiments at 20° are marked by crosses. Their results at higher CO_2 tensions more nearly fall on a smooth curve, but it is impossible to compare them with hydrogen-electrode determinations, because the hydrogen electrode is not very reliable in low partial pressures of hydrogen, even though a correction be applied for change in hydrogen pressure or concentration.

By means of the conversion table (fig. 6) it is possible to determine the CO_2 tension of sea-water from the temperature and P_H as read by means of the standard tubes and colorimeter described on pages 44–46. The

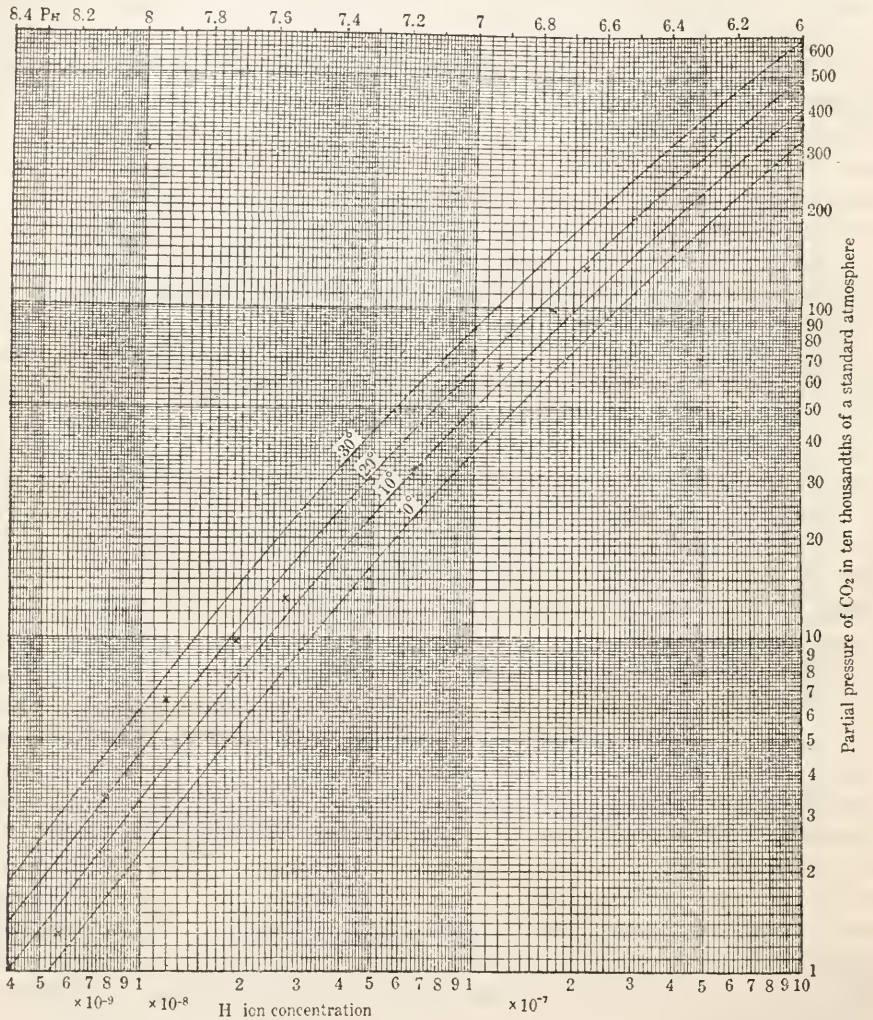


FIG. 6.—Conversion table for finding the CO_2 tension of sea-water from the P_H and the temperature. On the ordinate is measured the logarithm of the CO_2 tension in ten-thousandths of an atmosphere. On the abscissa is measured the P_H, with a scale below for converting it into the normality of H ions. The four curves are for 30°, 20°, 10°, and 0°, the last of which has been extrapolated. The crosses mark the determinations of Henderson and Cohn by the colorimetric method at 20°.

CO_2 tension is of first importance in the respiration of marine organisms. The rate of elimination of CO_2 by animals and its absorption by plants in the sunlight is directly proportional to the difference in CO_2 tension just outside and inside of the respiratory surfaces. Life in the sea is

more abundant in regions that are vigorously stirred, so that the CO_2 tension of a sample of the sea-water may be considered almost equal to that of the sea-water bathing the respiratory surfaces. The CO_2 tension has been one of the most difficult characteristics of sea-water to determine accurately.

It has been supposed that the amount of CO_2 in sea-water regulates the growth of seaweed, but the reverse is probably more nearly correct. The respiratory quotient of marine organisms seems to be about 0.7 to 1.0 and the respiration of animals and plants reciprocal. Some marine bacteria take their oxygen from nitrates, but this effect must be minute, since the supply of nitrates is small. The atmosphere can not be the chief regulator of the CO_2 of the sea, since there is about 30 times as much CO_2 in the sea as in the air. There is always a superabundance of CO_2 in sea-water to supply the needs of green, red, or brown seaweed, but by using it the plants increase the P_H of the water. It seems probable that the plants grow rapidly until the P_H that is most favorable to them is exceeded. This is in harmony with the fact that the P_H of the great oceans to the depth penetrated by light is more constant than the CO_2 tension, the P_H varying from about 8.0 to 8.25 and the CO_2 tension from about 0.00015 to 0.0005 atmosphere. The sea may be compared to the body of one of the higher vertebrates. The mammal regulates the P_H of the blood through the action of the respiratory center. The sea regulates the P_H of its surface-water most probably through the action of seaweed. The limit in the supply of oxygen probably prevents animal life from getting the upper hand temporarily and thus endangering the communal life in the sea.

It seems probable that seaweeds regulate the CO_2 of the atmosphere. The gaseous exchange between sea and air is necessarily at the surface and is comparatively slow. Bohr observed that the absorption of CO_2 from an atmosphere of the pure gas by CO_2-free water that is stirred (probably more vigorously than the sea ever is) is about 0.1 c.c. per square centimeter of surface per minute. Since the difference in CO_2 tension between air and sea seems never to exceed 0.02 per cent of that in Bohr's experiment, except in the polar regions, the rate of diffusion would not exceed 0.00001 c.c. per square centimeter per minute or 0.1 c.c. per square meter per minute in a storm, and necessarily much less in calm weather on account of the lessened rate of stirring at the surface. When we consider the volume of the sea and air compared to the sea-air surface, the fact becomes intelligible that the CO_2 in the air is relatively constant (3 per 10,000) in the different regions of the world where it has been accurately measured, whereas the CO_2 tension of the sea-surface varies from 1.5 to 5 per 10,000. The air is stirred more rapidly than the sea, and the CO_2 of the air seems to be determined by an equilibrium between gain in CO_2 over some regions of the sea surface and loss over others. The partial pressure of CO_2 in the

air is therefore the average CO_2 tension of the sea-surface. The burning of a billion tons of coal per year is probably changing the CO_2 content of the sea and of rocks and not of the atmosphere.

Regnard kept a tall open tube filled with a solution that became colored in the presence of oxygen at constant temperature to prevent convection currents. At the end of one year oxygen had diffused 4 meters deep into the solution. Although some oxygen was used in coloring the indicator, the experiment illustrates the slowness of diffusion in liquids.

Since both the conversion table for CO_2 content and the one for CO_2 tension are based on the assumption that the non-volatile buffer and the excess base in sea-water are constant, further work is being done on this subject. It seems probable that the weak bases in sea-water may assist the buffer action. The concentration of NH_3 is small, but is added to the total buffer value; it is non-volatile under the conditions present at the surface of the sea and is constantly being replenished by rain as it is used by organisms. Aluminium can act both as weak base and weak acid, but probably acts as a weak base in the sea. Organic acids besides CO_2 may be classed as non-volatile buffers. Their destruction in warm seas is probably due to the action of denitrifying bacteria, which catalyze the oxidation of organic matter with nitric and nitrous acids, as shown by Drew. The concentration of organic acids is probably maintained just below that which can be appreciably utilized by these bacteria.

The following artificial sea-water was found excellent for the growth of marine Protista and experiments in places where sea-water can not be obtained (the volumes being correct for 20°):

	Per liter.	m solutions.	Isotonic solutions.		Per liter.	m solutions.
	grams.	*c.c.*	*c.c.*		*grams.*	*c.c.*
$CaCl_2$, dry..	1.22	11.	(0.38 m) 29.0	Na_2SiO_3....	0.0025	.....
$MgCl_2 \cdot 6aq$..	5.105	25.16	(0.37 m) 67.9	$Na_2Si_4O_9$...	.....	0.005
$MgSO \cdot 7_4aq$..	7.035	28.55	(0.975 m) 29.5	H_3PO_4.....	.....	0.002
KCl.....	0.763	10.23	(0.577 m) 17.7	H_3BO_3.....	0.062	1.
NaCl.....	282.7	483.65	(0.568 m) 852.0	$Al_2Cl_6 \cdot 12aq$	0.026	0.01
$NaBr \cdot 2aq$...	0.0824	0.8	(0.565 m) 1.4	NH_3.....	.....	0.001
$NaHCO_3$....	0.21	2.5	(0.930 m) 2.7	$LiNO_3$.....	0.0014	0.002
				H_2O.....	(to 1 liter)	373.63

If Na_2SiO_3 is used, it should be dissolved in the H_2O before adding the other salts or it will be impossible to get into solution. We used a thick sirupy solution of water-glass (said to be $Na_2Si_4O_9$) and made an analysis of the Si concentration because we wished to ascertain the buffer value, but for the growth of organisms it is sufficient to consider the commercial solution as equivalent to 6 m SiO_2 or 1.5 m $Na_2Si_4O_9$, and the quantity desired should be obtained by successive dilutions, as a strong solution will precipitate when added to the other salts.

For plants at least it is permissible to add a drop of the strong solution per liter and allow it to precipitate. If cultures are grown in soft glass, no further additions of Si will be necessary to supply that used up by diatoms.

When sea-water is evaporated and redissolved oxides of manganese and iron, $CaSO_4$, SiO_2, $Ca_3(PO_4)_2$, $CaCO_3$, CaF_2, $Ca(BO_2)_2$, Al_2O_3, $Ba(BO_2)_2$, $BaSO_4$, and, under certain conditions, $MgNH_4PO_4$ are difficult to get into solution, but by adding HCl and evaporating, a paste containing gypsum will be obtained which will go into solution in about 3 days and may be neutralized and 0.21 gram $NaHCO_3$ per liter added.

Another method of determining the CO_2 tension on board ship or in laboratories without general chemical equipment was developed. The portable microapparatus for determining the total CO_2, described in section 4, was used and conversion tables were prepared for determining the CO_2 tension from the total CO_2, the temperature, and the excess base. Figures 7, 8, and 9 are conversion tables for finding the CO_2 tension of sea-water of 23, 24, and 25 excess base, respectively. Each curve is for the indicated temperature only, and the values for 0° were extrapolated. Tables for sea-water of any other excess base titration may be plotted with the aid of figure 10, which shows the change of CO_2 with change in excess base when other factors are constant.

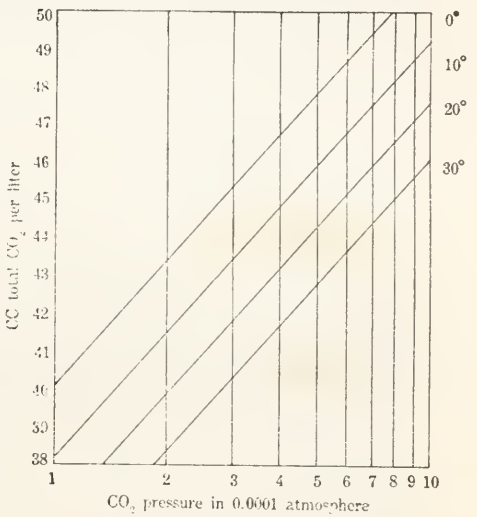


FIG. 7.—Conversion table for finding the CO_2 tension of sea-water of 23 excess base from the temperature and total CO_2. Each curve is for only one temperature, and that for 0° has been extrapolated. On the ordinate is given the total CO_2 and on the abscissa the CO_2 tension in ten-thousandths of an atmosphere.

Although it is theoretically a simple matter to determine the CO_2 tension directly, in practice it has been most difficult and attended with large experimental errors. Perhaps the most direct method is to shake a large quantity of the sea-water with a small quantity of air in a bottle until equilibrium is established, and analyze the air in the bottle. Errors arise in failure to reach equilibrium or to correctly determine the pressure and temperature or to correctly analyze the air. The analysis of one part of CO_2 in 10,000 parts of air may be done by the titration method, in which there are solubility and other errors, or by measuring the gas-volume contraction after absorption of CO_2, in which there are temperature, pressure, and meniscus errors. A

conversion table was made by Fox. For those who may be confused at first by logarithmic paper the relation of P_H and CO_2 tension is shown on ordinary coordinate paper in figure 11.

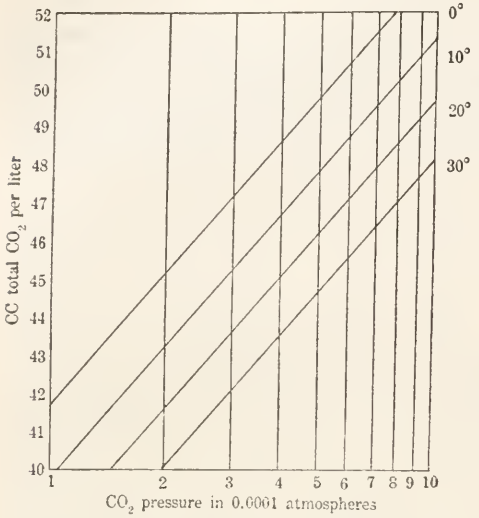


FIG. 8.—Conversion table for finding the CO_2 tension of sea-water of 24 excess base from the temperature and total CO_2.

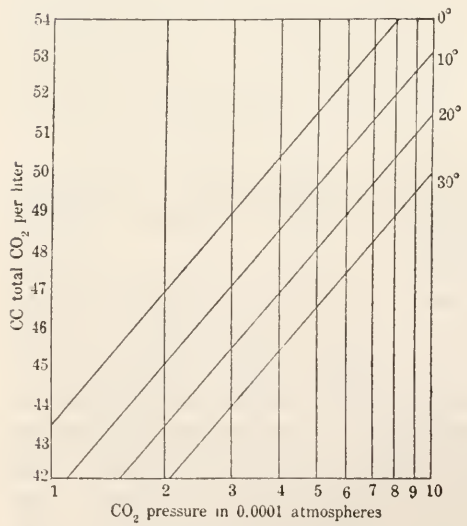


FIG. 9.—Conversion table for finding the CO_2 tension of sea-water of 25 excess base from the temperature and total CO_2.

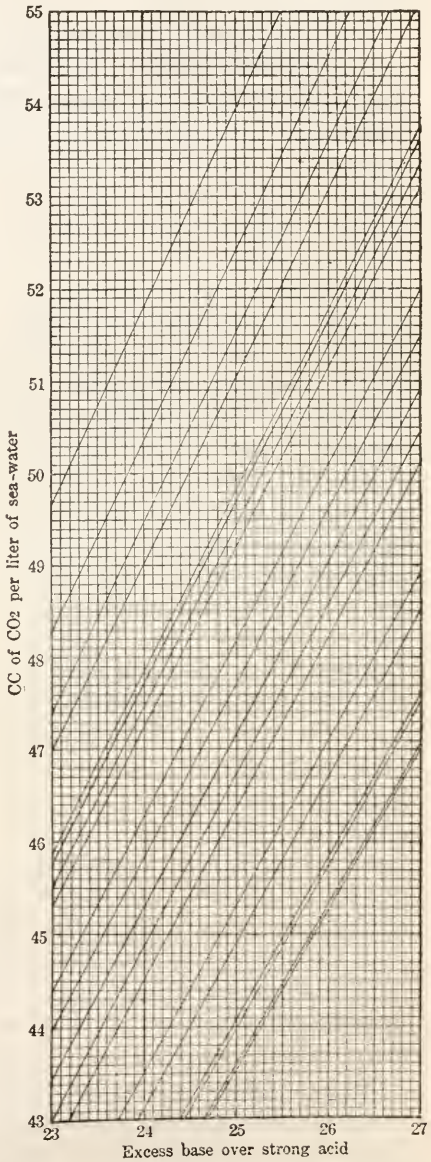


FIG. 10.—Data to be used in making a conversion table like fig. 9, but of any other excess base. Transfer values from fig. 9 to the 25 ordinate and draw diagonals through them parallel to the nearest diagonal in fig. 11; the values sought are found where these diagonals cut the ordinate of the excess base sought.

The determination of CO_2 of the air is made by placing about 10 c.c. of natural or artificial sea-water containing the thymolsulfophthalein in a nonsol test-tube of exactly the same bore as the sealed tubes and bubbling air through it to equilibrium and comparing with the tubes. The CO_2 tension of the air is read from figure 6. In order to hasten equilibrium the test-tube should be tall and have a series of constrictions above the portion necessary for colorimetry. A rubber stopper

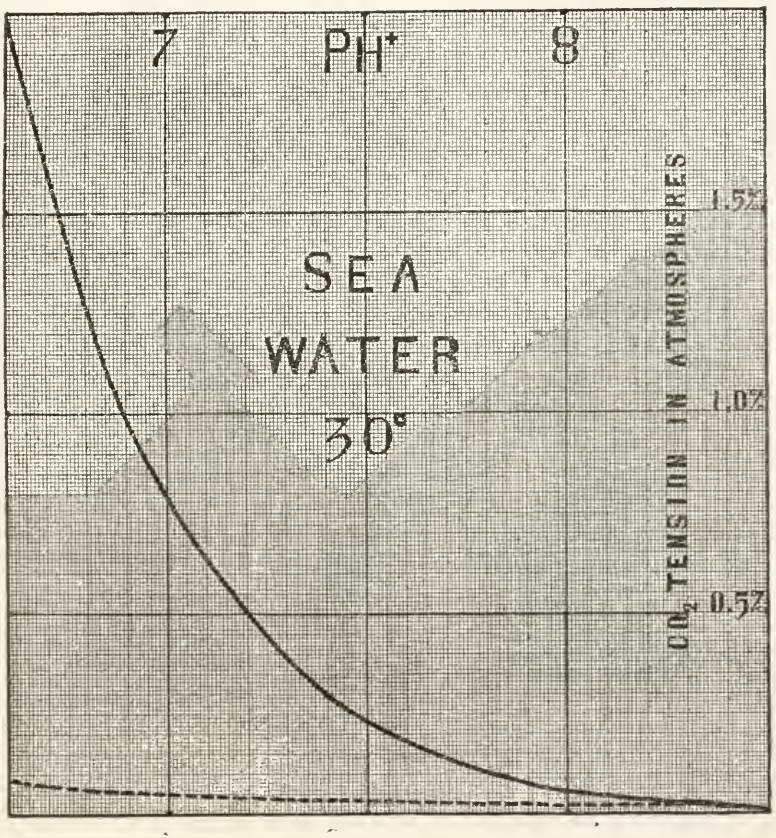


FIG. 11.—Conversion table for finding the CO_2 tension of sea-water at 30° from the P_H. This figure is explanatory of figure 6. The broken line is to be disregarded. (From Jour. Biol. Chem., 1916, XXVIII, 135.)

with 3 holes is inserted and a glass tube leading to the bottom, its closed end being pierced by fine holes; a tube is connected to a suction pump and a mercury manometer inserted in the third hole. The CO_2 content of the air is the CO_2 tension $\times 760 \div$ (the barometric reading $-$ manometer reading). Thymolsulfophthalein is decolorized by bubbling air through its solution for a long time, but this seems not to be an oxidation or a reduction.

CALIBRATION OF AN IMPROVED INDICATOR METHOD FOR USE ON SHIPBOARD AND IN POORLY EQUIPPED LABORATORIES.

After the excellent work of Sörensen and his associates on the indicator method it might be supposed that the last word had been spoken on this subject. But there are several disadvantages in Sörensen's method. The solutions have to be exposed to the air during the determinations and are placed in such a position that the operator's breath might blow directly into them. (This has been obviated in our method by sealing the tubes by fusing the glass, as done by Rowntree and his associates.) The salt-content of Sörensen's buffer mixtures is much less than in sea-water, and a large salt correction had to be applied, and no new indicators could be used until their salt error was determined. This has been obviated by calibrating new buffer mixtures of the same salt-content as sea-water. The use of a colorimeter that brings the centers of the tubes together in a sharp line makes it possible to compare the sealed tubes from the side even more accurately than open tubes viewed from above. The sealed tubes are gradually changed by solution of even the most resistant glass, but this change has been reduced by increasing the volume-surface ratio as well as the buffer and NaCl concentration. The selection of the brilliant sulfophthalein indicators, brought to our notice by the work of Lubs and Clark, greatly facilitates the determinations. We found that the salt error of all of these indicators so far calibrated is the same; at least no distinction could be made within the range that we had to use.

Although we made the salt-content of the buffer mixtures the same as sea-water, it is sometimes convenient to apply a slight salt correction for the slight variation in the salinity of ocean-water rather than carry a separate set of tubes for each salinity. But the magnitude of this correction seldom exceeds one-sixth of the correction that had to be applied to Sörensen's buffer mixtures. In our final set of tubes, a slight compromise was made in order to satisfy all of the principles set forth at once. Borax is the best buffer and is not very soluble; its solubility is reduced by the presence of salt. Since it was desired to have as high a concentration of borax as possible, the salinity of the buffer mixtures was made to equal that of sea-water of about the lowest salinity ordinarily met with, and the labels of the tubes were changed so as to correct for salt error of sea-water of the mean salinity that is to be encountered in an investigation, and directions were given for correcting for variation in salinity. Unless otherwise specified the tubes are labeled for a salinity of 31.25 grams per kilogram and with these tubes salinity variation within the ordinary range of the larger oceans causes slight errors that may be allowed for.

The buffer mixtures were made of different proportions of two stock solutions. In making a set of tubes, the indicator was selected and added to portions of the two stock solutions in two automatic burettes

in the proportion of 10 mg. per liter. The burettes were protected with soda-lime tubes and the condensation water was mixed up by shaking the burette occasionally. For this purpose a burette supported by the stock bottle is most convenient, since connections are not damaged in shaking. This burette is sometimes known as Squibb's automatic.

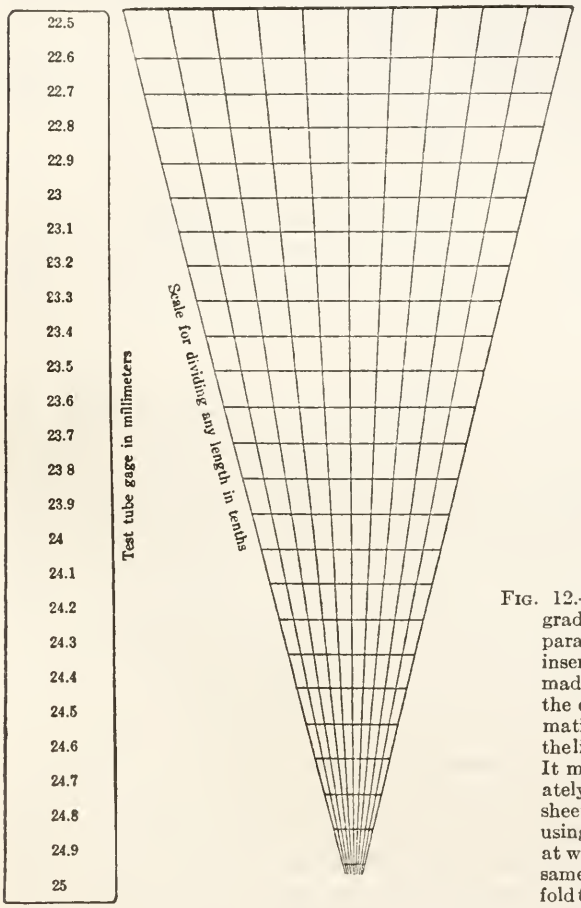


FIG. 12.—Test-tube gage and scale for graduating the special gasometric apparatus. The test-tube gage is to be inserted in the test-tube. Its slope is made very gradual in order to lessen the error produced by incorrect estimation of the position of the union of the lip and cylindrical part of the tube. It may be cut out and used immediately or after pasting it on a piece of sheet metal of the same shape. In using the other scale, find the position at which the width of the scale is the same as the length to be divided and fold the scale parallel to the heavy line.

A good glass as regards solubility is pyrex, but we have not become skillful enough to use glass of so high a melting-point. On account of its color Jena glass could not be used. The tubes used were nonsol test-tubes of 24 mm. bore. We obtained a very large number of these and divided them into grades in regard to bore, with the text-tube gage shown in figure 12. Those of the same bore and the most nearly cylindrical were selected for a set. The measurements were verified with a micrometer caliper, and no variation in any diameter greater than 0.2 mm. was allowed. Since there is danger of getting CO_2 into the tubes in sealing, they were first constricted in the middle to a very

narrow neck, filled, and quickly sealed by applying the blast lamp to the center of this constriction, the part removed being saved for use with sea-water. The blast lamp and all other flames were in a separate room from the burettes. The seal left a point on the tube liable to be broken, but this was covered with a layer of melted chewing-gum followed by a cap of sealing-wax. In the following list of mixtures the first column gives the percentage of the acid solution having 0.3 m boric acid and 2.25 per cent NaCl, the second column the percentage of the alkaline solution of 0.075 m borax and 1.9 per cent NaCl, the

1	2	3	4	5	6	7	1	2	3	4	5	6	7
Per cent boric.	Per cent borax.	P_H Cl = 13.9 per liter all indicators.	P_H Cl = 17.7 per liter sulfophthaleins.	P_H Cl = 21.5 per liter sulfophthaleins.	C.c. boric.	C.c. borax.	Per cent boric.	Per cent borax.	P_H Cl = 13.9 per liter all indicators.	P_H Cl = 17.7 per liter sulfophthaleins.	P_H Cl = 21.5 per liter sulfophthaleins.	C.c. boric.	C.c. borax.
79.5	20.5	7.50	7.45	7.40	23.90	6.1	47	53	8.30	8.25	8.20	14.10	15.9
78	22	7.55	7.50	7.45	23.4	6.6	44.5	55.5	8.35	8.30	8.25	13.45	17.55
76	24	7.60	7.55	7.50	22.8	7.2	42	58	8.40	8.35	8.30	12.60	17.4
74	26	7.65	7.60	7.55	22.2	7.8	39.5	60.5	8.45	8.40	8.35	11.85	18.15
72	28	7.70	7.65	7.60	21.6	8.4	37	63	8.50	8.45	8.40	11.10	18.9
70	30	7.75	7.70	7.65	21.0	9.0	34.5	65.5	8.55	8.50	8.45	10.35	19.65
68	32	7.80	7.75	7.70	20.4	9.6	32	68	8.60	8.55	8.50	9.6	20.4
66	34	7.85	7.80	7.75	19.8	10.2	29	71	8.65	8.60	8.55	8.7	21.3
64	32	7.90	7.85	7.80	19.2	10.8	26	74	8.70	8.65	8.60	7.8	22.2
62	38	7.95	7.90	7.85	18.6	11.4	23	77	8.75	8.70	8.65	6.9	23.1
60	40	8.00	7.95	7.90	18	12	20	80	8.80	8.75	8.70	6.0	24.0
58	42	8.05	8.00	7.95	17.4	12.6	17	83	8.85	8.80	8.75	5.1	24.9
56	44	8.10	8.05	8.00	16.8	13.2	14	86	8.90	8.85	8.80	4.2	25.8
54	46	8.15	8.10	8.05	16.2	13.8	11	89	8.95	8.90	8.85	3.3	26.7
52	48	8.20	8.15	8.10	15.6	14.4	8	92	9.00	8.95	8.90	2.4	27.6
51	49	8.22	8.17	8.12	15.3	14.7	4.5	95.5	9.05	9.00	8.95	1.35	28.65
49.5	50.5	8.25	8.20	8.15	14.85	15.15	1	99	9.10	9.05	9.00	0.3	29.7

third column gives the P_H, the fourth column the P_H of sea-water of S = 31.25 grams per kilo (Cl = 17.3 per kilo or 17.7 per liter) of the same color, and the fifth column the P_H of sea-water of S = 37.76 (Cl = 20.9 per kilo or 21.5 per liter) of the same color, the actual volume of acid solution used for a tube in the sixth column and of alkaline solution in the seventh column. Unless otherwise specified, the tubes are labeled according to the fourth column, and those of P_H 7.4 to 7.7 sterilized.

The first difficulty encountered was in obtaining a boric-acid solution with the same salt effect on the indicators as the borax solution. Starting from the conclusions of Palitzsch as to the salt value of boric acid, we finally developed the solution given. We have calibrated these tubes with sea-water about 50 times and in all parts of their range. Our tubes are labeled according to column 4 of the table, and include a set of o-cresolsulfophthalein tubes from P_H 7.45 to 8.30 and a set of thymolsulfophthalein tubes from P_H 7.9 to 9.05. The tubes deposit borax crystals when kept long at a low temperature and these crystals should be dissolved by warming the tubes before using them.

No special precautions are necessary in making the solutions. Reagent borax and boric acid needs no recrystallization, but commercial products must be recrystallized by dissolving in hot distilled water and filtering and cooling, then removing the mother liquor as much as possible and rinsing the crystals in ice-cold distilled water. The boric acid may be dried in a desiccator, but must not be heated above 70° to hasten the drying. Borax crystals effloresce in air on the surface, and powdery crystals must be discarded. It is usually sufficient to discard the surface layer of crystals from the bottle. The Na content of the borax may be titrated with 0.1 n HCl, using methyl red or dibrom-o-cresolsulfophthalein as indicator. It was found, however, that the eye was more capable of detecting efflorescence of the crystal before it was dissolved than by the use of methyl red afterwards. The other indicator is more sensitive and a flask containing the equivalent quantity of boric acid and NaCl may be used to get the end-point. The titration does not, however, distinguish between impurity and loss of water.

The borax solution may be prepared from boric acid and CO_2-free NaOH, but it takes more time to do this unless it is done at the same time that the solutions for determining the non-volatile buffer are prepared. Reagent NaOH contains more carbonate than is apparent on the surface of the sticks and must be purified. For this purpose, a saturated solution in distilled water is prepared (100 grams NaOH + 100 c.c. H_2O), and after thorough mixing it is sealed in a number of nonsol test-tubes by fusing the glass. These are placed upright in a thermos bottle (Dewar flask) or wrapped in cotton wool, in order to retard convection currents that stir up the precipitate. In 2 or 3 days the solution in the middle of each tube will be clear, the insoluble carbonate having settled, except for a little on the sides and the surface film. The only way to prevent a trace on the surface film is to completely fill the tubes, which can only be approximated. Owing to the high viscosity of the solution it can not be measured with delivery pipettes, and it is very convenient to have pipettes whose capacity is known (which can be determined by weighing when empty and when full of water and multiplying the difference by the density of water at that temperature). A tube is opened by cutting off the sealed end, the pipette tip is plunged to the center, and the sample quickly drawn. The meniscus is adjusted, the outside is wiped off, and the NaOH washed out of the pipette into a beaker with distilled water and titrated. If the opened test-tube is to be used again it must be quickly sealed with a lump of soft paraffin and inclosed in an air-tight container or CO_2-free compartment, preferably in a thermos bottle, for the disturbed carbonate to settle again; but it is advisable to use a tube only once. The solution of boric acid is prepared in a volumetric flask with distilled water freshly boiled in a metal container, but

enough space is left to admit the NaOH, which is run in before the flask is filled to the mark. It is usually necessary to adjust the NaOH concentration by successive titrations and dilutions with freshly boiled distilled water before it can be measured with sufficient accuracy. All solutions should be sealed or provided with soda-lime tubes. The sealed tubes of saturated NaOH gradually become contaminated by the solution of glass. This process is slower in the borax solution, but it should be kept in the best resistance glass available.

Our boric acid was recrystallized several times in quartz beakers and the reagent borax was titrated, to make sure of the proper Na content. We standardized our HCl solution with reagent $NaHCO_3$ that had been heated to 270° to 300° (short of sintering) by Dr. F. B. Kingsbury, using methyl orange as indicator and a CO_2 solution for standard color of the end-point of the indicator. The weights used were standardized and volumetric apparatus certified and used at constant temperature. All of these precautions are probably not necessary, however, to duplicate our results. Owing to the high concentration of the buffers larger absolute errors are permissible than in Sörensen's buffer mixtures. The boric acid can be titrated in borax with CO_2-free NaOH, using phenolphthalein as indicator, provided large quantities of neutral glycerol are added to prevent hydrolysis. 1 m NaOH titrates 1 m H_3BO_3 (Treadwell-Hall). We consider this titration superfluous, provided the crystals do not appear to have lost water, and especially if the Na content is correct. It is probably less expensive to obtain pure borax than to titrate it.

A little care is necessary in comparing the color of the tubes. If parallel rays of light are passed through them they act as lenses, concentrating the light to a line and making comparison difficult. If ground (sand-blasted) glass is used to disperse the light, the granular appearance prevents the most exact estimation of the color. We found the only satisfactory background to be a piece of very thin opal glass placed close behind the tubes. The middle portions of the tubes then appear uniform. In order to increase the accuracy of the observations, we removed the lenses from a stereoscope, placed their inner edges together, and fastened them at such a distance (8.5 to 10 cm.) in front of the tubes that when one eye was closed and the other held about 6 inches from the lenses, the centers of the tubes were brought together in a sharp line. Two colorimeters of this nature were made and proved satisfactory. The only serious difficulty we had was in making the illumination symmetrical. This is easily done by allowing direct sunlight to fall on the opal glass of the colorimeter and adjusting a pair of gun-sights (that have been placed for the purpose on the top of the instrument) in line toward the sun, but the eye is made unfit for colorimetric work for some minutes unless a dark glass is used. Owing

to the small part of the time during which we had sunlight, or even sufficient daylight, we tried mazda lights and found that we could compare o-cresolsulfophthalein (with some loss in accuracy) by means of a 250-watt stereopticon lamp, but this would not do at all for thymolsulfophthalein. The best artificial light we have tried is a 100-watt "daylite" mazda (blue bulb) with aluminium reflector, but we have great difficulty in making the light symmetrical, probably owing to irregularity in the thickness of the blue glass. This adjustment is made easier by a further dispersion of the light by means of a sand-blasted glass placed several inches behind the opal glass, but in every case it is necessary to verify a color-match by interchanging the tubes. The lamp was placed as close as possible to the tubes, but it might be supposed that the use of a larger lamp or thinner opal glass, thus enabling the lamp to be placed at a greater distance, would more nearly approximate the conditions with sunlight. We did not ascertain that the opal glass is colorless, although it appears pure white. The sand-blasted glass had a slight greenish hue noticeable on the edge, but we were unable to obtain colorless sand-blasted glass before the close of the experiments. The first attempt at using the colorimeter may lead to the supposition that the tubes may be matched more accurately by simply holding them before a piece of opal glass in the window, but this is due to failure of symmetrical lighting in the colorimeter. With a little experience it is possible to distinguish tubes in the colorimeter, which appear alike without it.

The indicators may be used in aqueous solution, since dilution of the sea-water has little effect on the P_H. In our experiments, however, it was necessary to use very concentrated solutions of the indicators, because we used the same sample of sea-water for the hydrogen electrode, the colorimeter, and the analysis of total CO_2, and dilution has an enormous effect on the CO_2 content at constant CO_2 tension. We made up 0.1 per cent solutions of the indicators in ethyl alcohol redistilled over sodium. The o-cresolsulfophthalein dissolved on heating, but the thymolsulfophthalein had to be assisted by a little CO_2-free NaOH. Enough NaOH to bring the color of the indicator to the middle of its range makes it more sensitive, but unless it is protected from CO_2 it will absorb quantities of this gas and become correspondingly insensitive. If NaOH is used to assist solution, it is safer to add an equivalent of HCl, as the small amount of this salt formed will not be appreciable when mixed with sea-water. Since we used only 0.1 c.c. of 0.1 per cent alcoholic solution of the indicator for 10 c.c. of sea-water, we do not believe the alcohol influenced the indicator method appreciably, but we suspect that both alcohol and indicator tended to depolarize the hydrogen electrode and increase the time necessary for equilibrium.

It is very difficult to completely prevent the concentration of the indicator solutions by evaporation while in actual use, and we did not attempt to do it. We kept the solutions in small Erlenmeyer flasks, each having a pipette, graduated in hundredths of a cubic centimeter, passed through the stopper into the solution. Perhaps the most convenient way of correcting the concentration of these solutions is to make up one tube of distilled water containing the same concentration of the indicator as the standard buffer mixtures, at the time the standard tubes are made. There is enough CO_2 in distilled water to turn these indicators to their maximum yellow color, yet not enough to carry them near their secondary color range (red in strong acid). The yellow color is constant over a very wide P_H range. It is then only necessary to determine the quantity of indicator solution necessary to color distilled water to the same degree of yellow as the sealed distilled-water tube, in order to have the amount necessary to put in the same volume of sea-water in determining the P_H. Even without this special tube, it is possible to determine colorimetrically (by repeated trials) the amount of indicator to add to sea-water; the color of each standard tube is the resultant of two colors (red and yellow or blue and yellow). It is impossible to match it with these colors in any other proportion, no matter how the concentration is changed. If the proper concentration of indicator is unknown, it may be found by repeated trials to match the color, using sea-water of any P_H within the range of the tubes. It was this consideration which led us to discard phenolphthalein and a-naphtholphthalein, since each showed only one color.

NEW APPARATUS FOR ELECTROMETRIC DETERMINATION OF HYDROGEN-ION CONCENTRATION AND FOR GAS ANALYSIS.

TECHNIQUE OF THE EXPERIMENTS.

The hydrogen electrodes were each made of a gold disk (later covered with a thin layer of palladium black) welded to a platinum wire sealed through the glass. There seems to be only one way to do this. A bit of gold is wound or folded over the end of the platinum wire and held in the flame until it just melts; then it is instantly removed. The congealed gold drop is then hammered on to the edge of the gold disk. The electrode is cleaned by filling the glass parts with a saturated solution of potassium bichromate in concentrated sulphuric acid (cleaning fluid) and rinsed thoroughly.

The palladizing solution is made by dissolving about 1 per cent of palladium chloride in distilled water and filtering. No reducing agent, such as basic lead acetate, is necessary. In fact, our solutions were weakened by spontaneous reduction, becoming dark with colloidal palladium; but this finally settled out, by further reduction, and the necessity of refiltering in order to watch the palladization was avoided.

The apparatus was filled with the palladium solution, a platinum wire was inserted for an anode, and a current of 4 volts passed until the gold became black. When necessary to repalladize, the apparatus was left full of nitric acid over night, which completely removed the palladium; it was then rinsed and the process was repeated. Palladium black does not seem to have the effective life of platinum black, but it was necessary to use it, as it can be removed without destroying the glass parts or the use of aqua regia. There is no necessity of electrolyzing H_2SO_4 in a hydrogen electrode, since any free chlorine is removed with the anode when it is taken out unless some aqua regia has been formed in it. If the palladium black is deposited too rapidly it will not stick.

We do not understand why a freshly palladized electrode behaves in a more reliable manner than one which has soaked in distilled water for many days. The electrode is thoroughly charged with hydrogen when palladized, but much of this is probably lost during the rinsing. Nitric and, to a smaller extent, other mineral acids attack palladium black and oxidizing agents probably affect it; hence we never cleaned palladized electrodes with cleaning fluid. It seems probable that the alcohol and indicator were slowly reduced by hydrogen at the surface of the palladium black, although we did not detect any decrease in the indicator concentration. It is probable that most of the alcohol had been removed from the solution by the current of gas before our final readings were taken. Such suppositions as to the effect of alcohol were based on variations of the potential of the electrode, and it is well known that such occur whenever free oxygen or oxyhemoglobin, that are reduced at the electrode, are present. The phenomenon is probably general, the ease with which the substance is reduced probably determining merely the rate at which reduction occurs. Even with a small amount of free oxygen in the solution, we found it possible to obtain the correct result by tilting the apparatus so that the platinized or palladized disk communicated with the main mass of fluid by only a thin film.

The oxygen is apparently used up in the immediate vicinity of the electrode in a relatively short time and the proximity to a large store of hydrogen hastens the resaturation of the electrode. But because of our ignorance of some of the factors involved, we did not wish to rely on any such assumptions in this set of experiments, and consequently we made about half of the determinations soon after immersion and half soon after removal of the electrode by tilting, and took the mean value. In some cases it was necessary to apply corrections for pressure of CO_2 and H_2 when they were not exactly the same in the gas and water phases, but these differences never exceeded 1 per cent of the partial pressure of the gas. We were very much impressed with the slowness with which equilibrium is established between the solution and the gas above it.

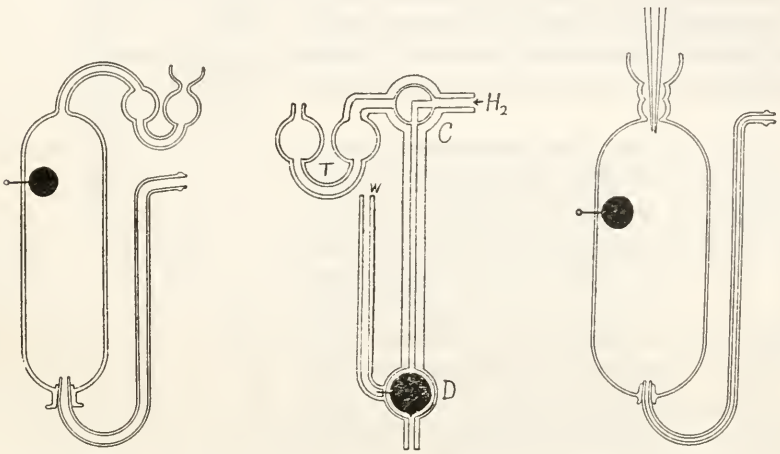
An experiment designed to illustrate this was made as follows: 5 c.c. of sea-water were placed in the hydrogen electrode and a stream of pure hydrogen of about 100 c.c. per minute passed through it. About 15 minutes were required for saturation of the water with hydrogen (so that the P_H was the same, 8.2, as with stagnant hydrogen, but with the electrode raised out of the water), and this time and 10 minutes more were counted out of the experiment. The water now contained 0.2 c.c. of CO_2 in the form of carbonates and bicarbonates, calculated from the P_H of 8.3. In 10 minutes, with the passage of 1,000 c.c. of H_2 in small bubbles, the P_H rose to 8.4, indicating that 0.007 c.c. of CO_2 had been removed. The rate of removal constantly decreased in an asymptotic curve, and at the end of 2 hours a P_H of 9 was reached, indicating that a total of 0.05 c.c. of CO_2 had been removed. During the next 10 minutes the P_H rose to 9.02, indicating the further loss of 0.0014 c.c. of CO_2. At the end of 270 minutes the P_H had reached 9.18 and was rising at the rate of 0.01 in 10 minutes, indicating a total loss of 0.0616 c.c. and a final rate of 0.0007 c.c. per 10 minutes.

There was still enough CO_2 in the sea-water to convert most of the excess base into normal carbonate. This shows the hopelessness of reaching equilibrium at very low CO_2 tensions, and we seldom attempted anything lower than 0.0002 atmosphere, and then made tests to see that equilibrium was attained within the limit of error of measurement. The buffer mixtures were intended to be CO_2-free, and similar experiments on them showed that if traces of CO_2 were in them they were not removed by hours of bubbling hydrogen through them.

We know of no attempts to determine the asymptote or limit that is approached in the above experiment. If it is an NaOH solution that is approached, we should be able to remove any traces of CO_2 from the buffer mixture (theoretically with ideal apparatus). The weak acid in the buffer should help displace CO_2. Perhaps the reason we could not reach a higher P_H by bubbling H_2 through the buffer mixture was that the traces of CO_2 were not sufficient to measurably change the P_H. The electrodes for comparing the colors of the buffer mixtures and sea-water of the same P_H were made of the same bore as the sealed tubes and so that they could be placed in the colorimeter after determining the P_H electrometrically. The essential form of these is shown in figure 13. There are no ground joints or rubber connections exposed to the air. The hydrogen is allowed to escape through the trap at the top, which is filled with some of the same solution as in the electrode. Electrolytic connection with the calomel electrode is made through the ground joint at the bottom submerged in an intermediate vessel of saturated KCl solution.

Some of the electrometric titrations were made with the dipping electrode shown in figure 14, which is rinsed with the solution by

allowing the solution to rise in it by gravity and blowing it out with hydrogen. It is most convenient to make the vessel for holding the sea-water with a ground joint at the bottom for electrolytic connection. Select a glass-stoppered bottle, whose stopper does not leak, and cut off the bottom. This is done by cutting a short groove with a file or glass knife, and holding a heated rod just beyond each end of the cut until a crack is started. One heated rod is moved over the line chosen for the cut, at such a rate that the crack follows the rod. If metal rods



13 14 15

FIG. 13.—Hydrogen electrode that can be placed in the colorimeter for calibrating the colorimetric method of determining the P_H, being of the same bore as the sealed colorimetric tubes. The ground joint at the bottom should not be greased, as it is for the electrolytic connection with the calomel electrode through the interposition of a KCl bath. The black circle is a palladized or platinized gold disk welded to a platinum wire fused through the glass. The long narrow tube is for the introduction of a continuous stream of hydrogen which escapes at the top through a trap which prevents the backward diffusion of air.

FIG. 14.—Dipping hydrogen electrode for titrations. Insert a drop of Hg and copper wire in *W*. The bulb *D* is immersed in the fluid in an inverted glass-stoppered bottle with the bottom cut off. The ground joint at the stopper serves for electrolytic connection with the KCl bath. The cock *C* is turned so that the bulb *D* fills by gravity with the fluid, which drives out the hydrogen at the trap *T*. Hydrogen is allowed to bubble through again and the fluid allowed to rise until it just touches the palladized gold disk shown in black, when the reading is taken. (From Jour. Biol. Chem., 1916, XXVIII, 135.)

FIG. 15.—Hydrogen electrode for CO_2-free electrometric titrations. The principles of construction are the same as in figure 13, except that the trap at the top is made to admit a burette tip, and can be sealed with a drop of distilled water after the burette tip has been inserted so as to leave only a very narrow opening.

or large nails can not be obtained, pieces of glass heated to a red heat on the end will do. After removal of the bottom the bottle is inverted and immersed to the neck in the KCl vessel. In making the titration, the sea-water is pipetted into the bottle and covered with a paraffined cardboard with holes for the electrode and burette tip.

A more convenient form of titration electrode is shown in figure 15. It has the advantage that the air is excluded from the whole solution by

lowering the burette tip far enough into the hole in the top to seal with a water seal. Hydrogen is passed through for about 15 minutes before commencing the titration and is continued all through the titration, escaping through the water seal, which should be replenished with a drop of distilled water in case it is blown out by the hydrogen.

The most difficult problem was to bring the sea-water in the hydrogen electrode to a known CO_2 tension. Various forms of apparatus for treating the sea-water with gas mixtures were tried, but it was found that a very long time was required to reach equilibrium. We finally devised a simple apparatus in which equilibrium could be reached in 30 minutes or less (although we took more time to make sure of it after the first time tests were made). This tonometer electrode (fig. 16) consists of a tube 450 mm. long and 7 mm.

16

FIG. 16.—Tonometer electrode for determining the P_H of sea-water at a predetermined CO_2 tension. A mixture of CO_2 and H_2 is bubbled through the sea-water in a narrow tube; then the stopcocks are closed and the electrode inverted so that the sea-water flows into the wide portion, which is of the same bore as the colorimeter tubes, so that the hydrogen electrode determination of the P_H may be compared with that by the colorimetric method. The stopcock on the wide end of the electrode is not greased, as it serves for electrolytic connection with the KCl bath.

17

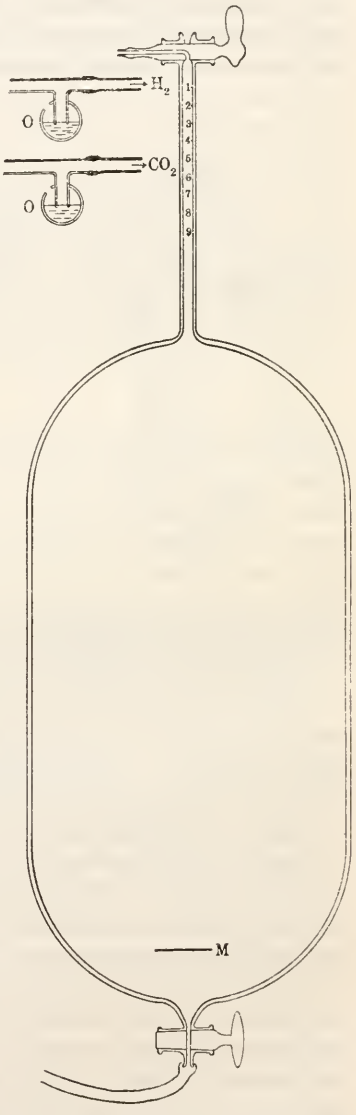


FIG. 17.—Gasometer for mixing CO_2 in ten-thousandth parts with H_2 (atmospheric pressure being insured by the traps, *OO*). The apparatus holds 25 pounds of Hg and hence must be bound with iron wire run through boiling sealing-wax while being wound on diagonally. The narrow tube is graduated in tenths or hundredths of a cubic centimeter. The apparatus holds 1 liter of gas when the mercury meniscus is at *M*. The proportions are not quite correct in this figure. It is best to have the apparatus narrower and so that *M* may be marked on the cylindrical part; or (as we accomplished in one apparatus) *M* may be at the lower stopcock. The lower stopcock is connected by means of a piece of pressure rubber tubing to a leveling bulb held up by a rope passing through pulley attached to the ceiling.

bore with a stopcock at one end and the other end fused to a short tube of exactly 24 mm. bore, containing the palladized gold disk and closed with a minute ungreased stopcock. About 11 to 12 c.c. of sea-water containing the indicator were sucked up into it and it was clamped with the small tube downward (which then contained a column 30 cm. high of sea-water). The mixture of CO_2 and H_2 was passed in at the bottom in small bubbles, and the flow regulated so that the sea-water did not rise into the large tube. The gas mixture escaped at the top through a trap connected to the stopcock by a short rubber tube, to prevent the backward diffusion of air. The gas pressure of the bubbles varied from the barometer reading to the barometer + 300 mm. of water, the average being barometer + 150 mm. of water or about barometer + 11.5 mm. of mercury.

We tested equilibrium by first starting with sea-water of a known lower CO_2 tension than the gas mixture, and making a second determination on sea-water of a known higher CO_2 tension than the gas mixture. If the final P_H was the same in both cases we concluded that equilibrium had been sufficiently approximated, but in all later experiments we about doubled this time.

In making the electrometric reading the electrode was simply inverted so that the sea-water ran down into the large tube, the ungreased stopcock immersed in the KCl bath, and a platinum-tipped wire hooked into the platinum loop to connect to the potentiometer. If the reading was taken with the disk out of the water, the H_2 and CO_2 partial pressures were the same as in the original gas mixture, but if the disk was immersed, 11.5 was added to the H_2 pressure and $p\left(\frac{11.5}{\text{bar}}\right)$ was added to the CO_2 pressure in making the calculations. The pressures were always corrected for vapor tension of water before the final calculations.

The gas mixture was made in the apparatus shown in figure 17, holding about 25 pounds of mercury. This consisted of a liter separatory funnel with a narrow tube and 3-way cock attached at the top, and wrapped with wire passed through hot sealing-wax to increase its strength. By graduating the narrow tube in tenths of a cubic centimeter and making a mark, *M*, near the lower end of the separatory funnel to denote a total volume of a liter, it was possible to measure the CO_2 in parts per 10,000. The CO_2 was washed with $NaHCO_3$ solution and the H_2 with $HgCl_2$ solution and again in H_2O in wash bottles shaped like pyknometers, and passed through traps (*O*, fig. 17) to insure atmospheric pressure. The apparatus was filled with mercury by raising the leveling bulb by means of a rope passing through a pulley in the ceiling. The CO_2 apparatus was attached to the 3-way cock and the air was washed out of the connections. The 3-way cock was now turned as in figure 17, so that CO_2 entered the narrow tube when the

leveling bulb was lowered and the lower stopcock of the separatory funnel was opened cautiously. When the required amount of CO_2 was admitted, the lower cock was closed, the 3-way cock reversed, and the H_2 apparatus substituted. The CO_2 was washed out of the connections and the 3-way stopcock turned as in figure 17 and H_2 admitted by opening the lower stopcock. The operator kept his hand on this stopcock until the filling was complete, because the H_2 came faster at first than later, and there was danger of the mercury leaving the separatory funnel faster than the H_2 was supplied, in which case water would be sucked in from the trap *O*. It was necessary to allow a little H_2 to escape continuously through the trap *O*, as a matter of safety. When the

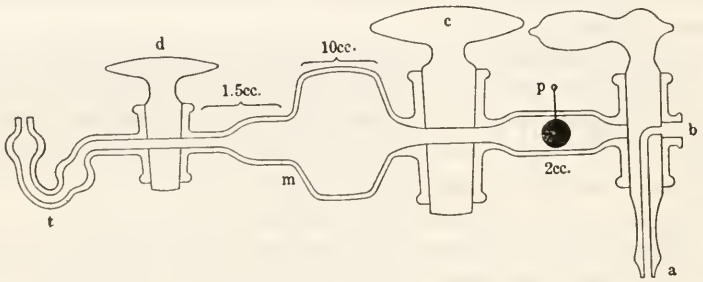


FIG. 18.—Hydrogen electrode for determining the P_H of water taken directly from the sea. A drop of water is placed in the trap *t*, to seal it, and the electrode is filled with pure hydrogen. The hole in the 3-way cock is filled with sea-water by sucking on a rubber tube attached to *b* while *a* is immersed in the water; then this cock is reversed and the suction applied at *t* until the sea-water rises to the mark *m*, when all cocks are closed. The 1.5 c.c. of hydrogen remaining in the electrode is shaken with the 10 c.c. of sea-water in the large compartment until it has the same CO_2 tension as the sea-water, when it is passed into the small compartment by opening the large cock *c* (which must be 4 mm. bore and not greased) and tapping the electrode. *D* is opened a moment before *c* is closed to reestablish atmospheric pressure and the electrode is shaken again before taking the reading. Electrolytic connection is made through the ungreased cock *c*. A wire is hooked in *p* to connect with the potentiometer.

mercury meniscus reached the lower mark, the cocks were closed and the apparatus shaken, the remaining mercury stirring and mixing the gas. The tonometer electrode was now attached, the leveling bulb was raised, and the gas mixture was passed through the tall column of sea-water in fine bubbles, which was accomplished only after a careful adjustment of all the stopcocks.

For the determination of the P_H of sea-water taken directly from the sea, the electrode shown in figure 18 was used. It was first filled with H_2 and then sea-water was admitted through *a* to the mark *m*, after washing out the air at *b*. The H_2 was shaken with 10 c.c. of sea-water in the large compartment; then by tapping the apparatus it was caused to enter the smaller compartment and again shaken. In this way the loss of CO_2 by the second portion of sea-water was minimized. The

ungreased stopcock was immersed in the KCl and the reading was taken in the usual manner.

The calomel electrodes were made from mercury redistilled in Hulett's still (and plated on platinum so as to be portable), KCl was recrystallized many times in quartz beakers, and calomel was made by the electrolytic method of Lipscomb and Hulett. The 0.1 n KCl calomel electrodes were made at 20° and hence have a very slight error at 10° and 30° due to volume change of the KCl solution in them. The KCl was weighed with standard weights and the weighings reduced to vacuo. A saturated KCl calomel electrode was often used as an intermediate on account of its lower resistance, but each time was compared against the 0.1 n electrode. The saturated electrode often

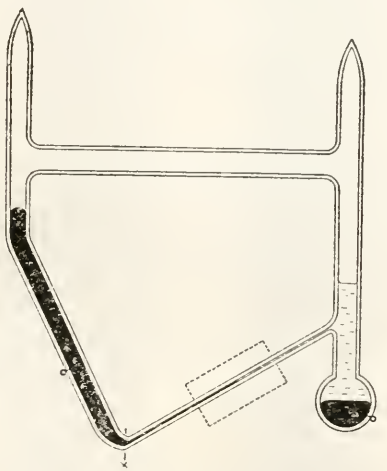


FIG. 19.—Capillary electrometer of increased sensitivity. The large bulb is inclined toward the observer by bending the neck while the glass is soft, so that the electrometer will lie flat on the stage of a microscope. It is attached in this position by means of rubber bands and the microscope is tilted back until the tube is horizontal. Wires are hooked in the little rings, which are platinum loops fused through the glass. The dotted rectangle represents a cover-glass mounted on the capillary with Canada balsam. The capillary must be the smallest obtainable and of thin enough walls to admit of an 8 mm. objective and x 20 ocular in the microscope. A few specks of dust in the ocular will serve to locate the zero-point, and a micrometer scale is not necessary.

varied about 1 millivolt, although the readings were never taken in less than 3 hours after the room was brought to constant temperature, after a maximum fluctuation of about 2 degrees.

Two special potentiometers made by Leeds and Northrup were used. One was standardized by the Bureau of Standards and the other compared with it. One of these is now at Tortugas and the other in Minneapolis. The special features are the time-saving arrangement and key which automatically short-circuits the capillary electrometer when not in use.

We had difficulties with the ordinary forms of capillary electrometers, but found the slight modification shown in figure 19 satisfactory. It was always necessary to make sure the electrometer was not polarized before taking a reading. The sensitivity was made sufficient by using the smallest capillary tubing obtainable and a microscope of very high power, especially in the ocular. The electrometer was attached to the stage of a tilted-back microscope with rubber bands. If polarized, it is tilted sidewise until a drop of mercury

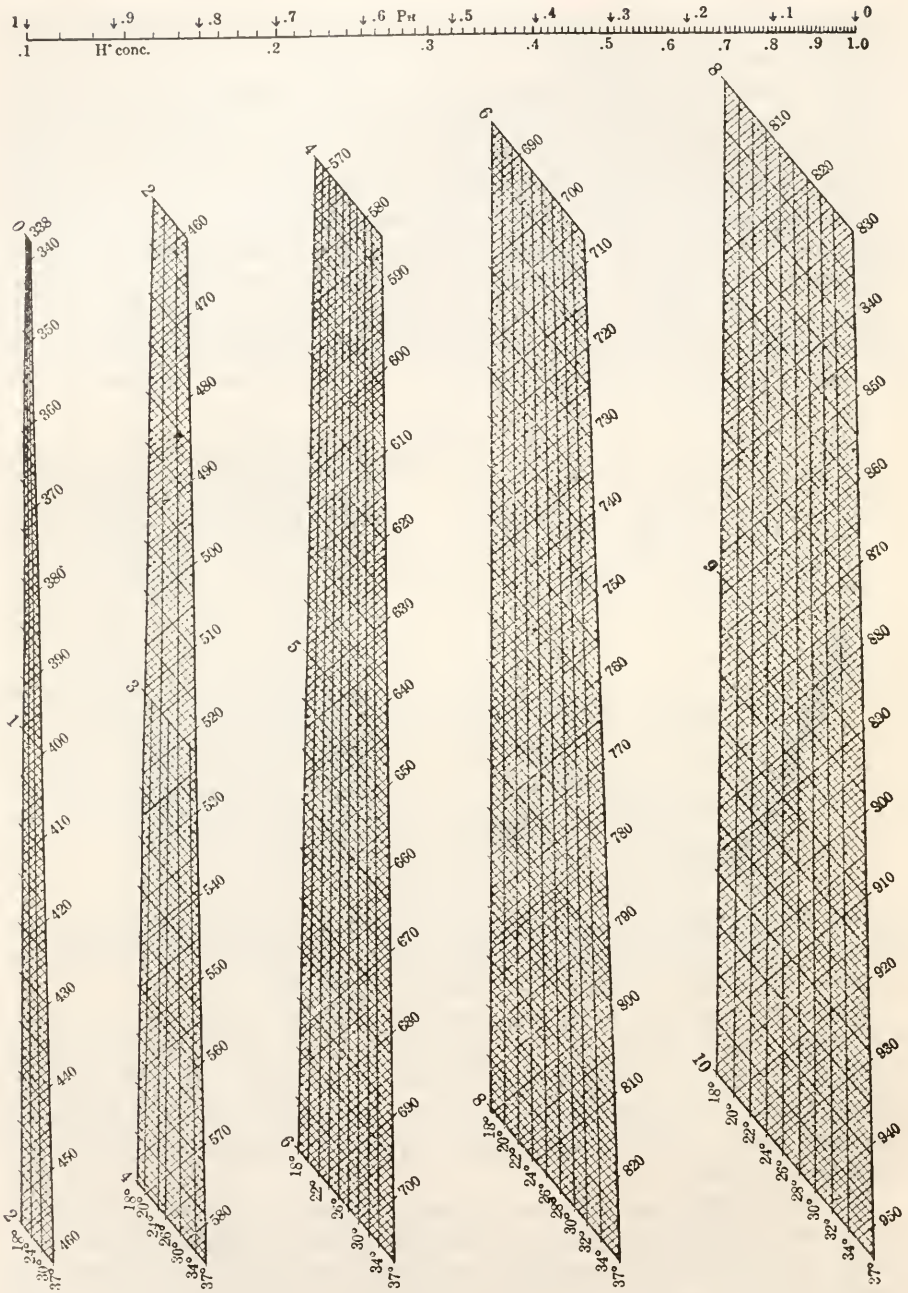


FIG. 20.—Table for conversion of millivolt readings into P_H when using a hydrogen electrode connected with a 0.1 n KCl calomel electrode. A correction for the concentration of hydrogen gas must be added to millivolt reading before using the table. The large figures from 0 to 10 denote P_H, the figures from 338 to 950 denote millivolts, and the temperature lines are designated at the bottom. The double scale at the top is for conversion of the fractional part of P_H into the digits used in the decimal system, the decimal-point being determined by the integral part of the P_H. We see no advantage in the decimal system over the logarithmic (P_H) system of expressing H-ion concentration, but have added this scale for comparison of results expressed in the two systems. In the upper double scale the arrow-heads denote tenths of a P_H and the lower scale the digits of the decimal system. Thus $P_H = 8.7$ is about the same quantity as H conc. $= 0.2 \times 10^{-8}$. P_H 7 was misplaced.

falls out of the capillary. A cover-glass is mounted on the capillary with balsam.

Before using the conversion table for converting millivolts into P_H a slight correction given in the following table was added to the millivolt reading, on account of the rarefaction of the hydrogen.

Barometer.	10°					20°					30°				
	Per cent of hydrogen.					Per cent of hydrogen.					Per cent of hydrogen.				
	100	99	98	95	90	100	99	98	95	90	100	99	98	95	90
780	0.1	0.2	0.3	0.6	1.3	0.9	1.0	1.2	1.6	2.3	1.7	1.8	1.9	2.3	3.0
770	0.3	0.4	0.5	0.8	1.5	1.1	1.2	1.4	1.8	2.5	1.9	2.0	2.1	2.5	3.2
760	0.5	0.6	0.7	1.0	1.7	1.3	1.4	1.6	2.0	2.7	2.1	2.2	2.3	2.7	3.4
750	0.7	0.8	0.9	1.2	1.9	1.5	1.6	1.8	2.2	2.9	2.3	2.4	2.5	2.9	3.6
740	0.9	1.0	1.1	1.4	2.1	1.7	1.8	2.0	2.4	3.1	2.5	2.6	2.7	3.1	3.8
730	1.1	1.2	1.3	1.6	2.3	1.9	2.0	2.2	2.6	3.3	2.7	2.8	2.9	3.3	4.0

The above table is calculated on the hypothesis that the potential of the hydrogen electrode in a normal solution of H ions depends on the concentration and not solely on the pressure of the hydrogen. Hence the value of the hydrogen electrode against the 0.1 n KCl calomel electrode is not 337 millivolts as used in case no correction for hydrogen pressure has been applied, but a slightly higher value. The average of the determinations listed by Clark and Lubs for temperatures up to

Table for converting millivolts into P_H *at 10°.*

P_H	+0.0	+0.1	+0.2	+0.3	+0.4	+0.5	+0.6	+0.7	+0.8	+0.9
3	506.45	512.1	517.7	523.3	528.9	534.5	540.1	545.7	551.3	556.9
4	562.6	568.2	573.8	579.4	585.0	590.6	596.2	601.8	607.4	613.0
5	618.75	624.4	630.0	635.6	641.2	646.8	652.4	658.0	663.6	669.2
6	674.90	680.5	686.1	691.7	697.3	702.9	708.5	714.1	719.7	725.3
7	731.05	736.7	742.3	747.9	753.5	759.1	764.7	770.3	775.9	781.5
8	787.20	792.8	798.4	804.0	809.6	815.2	820.8	826.4	832.0	837.6
9	843.35	849.0	854.6	860.2	865.8	871.4	877.0	882.6	888.2	893.8

Table for converting millivolts into P_H *at 30°.*

P_H	+0.0	+0.1	+0.2	+0.3	+0.4	+0.5	+0.6	+0.7	+0.8	+0.9
3	518.4	524.4	530.4	536.4	542.4	548.4	554.4	560.4	566.4	575.4
4	578.5	584.5	590.5	596.5	602.5	608.5	614.5	620.5	626.5	632.5
5	638.6	644.6	650.6	656.6	662.6	668.6	674.6	680.6	686.6	692.6
6	698.7	704.7	710.7	716.7	722.7	728.7	734.7	740.7	746.7	752.7
7	758.8	763.8	770.8	776.8	782.8	788.8	794.8	800.8	806.8	812.8
8	818.9	824.9	830.9	836.9	842.9	848.9	854.9	860.9	866.9	872.9
9	879	885	891	897	903	909	915	921	927	933

40° (which is the highest necessary in biological work) is 338.15, and we have rounded off this value of 338 for all temperatures between 0° and 40°.

After adding the correction from the above table, the millivolt reading was converted into P_H by the conversion table in figure 20, which was cut into five sections to save space. The larger numbers (1–10) denote P_H, the numbers 338 to 950 denote millivolts, and the vertical lines temperatures, as indicated below. The double horizontal scale above is for converting the fractional part of the P_H into the coefficient used in expressing the hydrogen-ion concentration, tenths of a P_H being denoted by the arrows above, and the coefficient by the finely divided scale below.

The table at 10°, near bottom of page 57, serves to extend the range, and the table at 30°, just below, serves as a guide to figure 20.

In choosing the size of the apparatus for the analysis of the total CO_2 in the sea-water, it was necessary to consider the fact that we had to bring the water sample to a known CO_2 tension before the analysis. We found it impossible to make more than a liter of the gas mixture correctly. We made gasometers on a larger scale, but they were unsatisfactory. It was also impossible to bring a large sample of sea-water to a known CO_2 tension with only 1 liter of gas mixture. We finally chose the micro-apparatus used by Van Slyke in determining the total CO_2 in blood-plasma. The large compartment of our apparatus held 50 c.c., but we would recommend its increase to 100 c.c. The form we used in the first part of the work was essentially like figure 21, but later we cut off the absorption pipette *P*, and absorbed the CO_2 by running NaOH solution directly into the gas burette *B*.

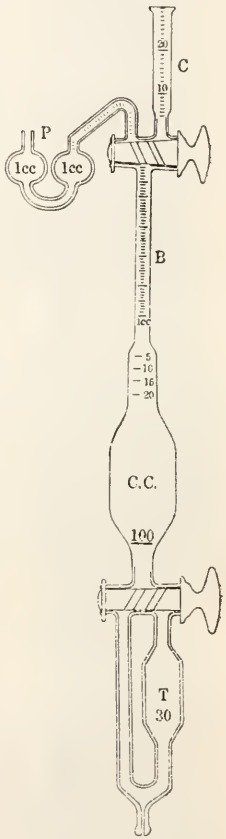


Fig. 21.—Van Slyke plasma CO_2 apparatus as used for the determination of the total CO_2, O_2, and N_2 of sea-water. The absorption pipette *P* may be omitted and a simple stopcock used on the top. A piece of small-bored suction rubber tubing, 80 cm. long, is attached to the lower end of the apparatus, and connected to a leveling-bulb with cylindrical sides that is held up by means of a swivel attachment to a screw passed through a nut held in a burette clamp attached to a tall rod support for the whole apparatus. The essential feature of the apparatus is the graduation of the gas burette *B* in hundredths of a cubic centimeter, so that the gas-volume can be accurately determined with the apparatus in the upright position and an upturned water meniscus below the gas. The graduations should go at least half-way around the gas burette to avoid parallax errors. It should be possible to estimate thousandths of a cubic centimeter by means of a lens.

The following description applies to the 50 c.c. apparatus without the absorption pipette. We would recommend the substitution of a simple 2-way stopcock for the one at the top.

The apparatus was clamped upright to a vertical iron rod rising 6 feet from the floor, and a horizontal rod was placed under the lower stopcock to prevent it slipping out of the clamp. The ordinary clamps used in chemistry were used, and the vertical rod supported in the middle by attaching it to the edge of a table with a staple, and at the bottom by inserting it into a hole in a board nailed to the floor. A leveling bulb with cylindrical sides was attached by means of soft-rubber suction tubing (1 mm. bore and 800 mm. long) to the lower end of the apparatus. The leveling bulb was attached by means of a swivel to a screw (of about 6 mm. pitch) and passing through a nut held in a burette clamp on the rod. Another burette clamp was attached to the rod about 800 mm. lower down, and the nut could be changed from one clamp to the other in a few seconds. The stopcocks were greased with a mixture made by dissolving pure gum-rubber (containing no sulphur) in boiling paraffin, and thinning with vaseline. This preparation was satisfactory at 10° and at 20°, but a more satisfactory preparation at 30° may be prepared from sapota-tree sap.

The apparatus was completely filled with mercury and exhausted once by closing the upper stopcock and lowering the leveling bulb, in order to practically free it from air. The bulb was raised again and the air-bubble forced out of the top. The mercury in the cup *C* was allowed to run down until it filled only the capillary neck. The leveling bulb was lowered below *B* and 10 c.c. of sea-water were allowed to run quickly from the tonometer electrode into the cup *C*, and then quickly into the burette *B*, care being taken that the capillary neck of *C* remained full of sea-water. 1 c.c. of 2 n HCl (*i. e.*, acid with about the same absorption coefficient for the elementary gases as the average sea-water) was introduced into *C* and allowed to run down into *B*. A little mercury was placed in *C* and some of it was allowed to run down into *B*, so that the hole in the stopcock was filled with it and closed. If the stopcock had two holes, as in the figure, the other hole and connecting tube had already been filled with mercury in filling the apparatus with mercury. This mercury effectively sealed the middle of the stopcock, and it seldom leaked, especially if more grease was smeared over the two ends of the stopcock. The stopcocks were usually greased again for each determination. Air getting in at first makes no theoretical difference, but leakage may ruin the determination by occurring at the last.

By lowering the leveling bulb, the mercury meniscus was brought to the 50 c.c. mark (100 c.c. in fig. 21). The lower cock was closed and the apparatus removed from the clamp and held with the left hand at the attachment of the rubber tube and the right hand at the upper tip

of the cup *C*, and shaken violently laterally while in the vertical position, for 2 minutes exactly. In this way the sea-water was brought into approximate equilibrium with the gas pumped out of it. The apparatus was clamped again and the lower cock turned so that the sea-water passed into the trap *T*, the screw being adjusted so that the water meniscus was brought exactly to the upper entrance to the hole in the lower stopcock. One minute was allowed for the sea-water to drain down; then the lower stopcock was reversed and the leveling bulb raised so that the gas in the gas burette *B* was brought to atmospheric pressure. One minute was allowed for the moisture caught between the glass and the mercury to rise, and then the sea-water on top of the mercury was carefully measured and recorded. It should be about 0.05 c.c. The leveling bulb was then raised sufficiently to make the mercury meniscus rise half the height of the water column. The lower stopcock was closed and the leveling bulb brought back to the same level as the mercury in *B*.

The apparatus was now removed from the clamp and placed horizontally, with the trap *T* upwards. By little jerks the mercury was forced into *B*, thus displacing the gas and water into the wide part of the apparatus. It was shaken in this position 1 minute, and returned to the clamp, care being taken that no water worked around from *T*, and rose into *B* when the lower cock was opened again. On opening this lower cock, the mercury meniscus should not rise or fall more than a small fraction of a millimeter. The leveling bulb was now carefully adjusted with the screw so as to bring its meniscus on exactly the same level as the mercury meniscus in the burette *B*. This is not at first an easy matter, but may be assisted by placing the leveling bulb directly behind the burette and placing 2 specks of dust on the mercury in it, so that it is possible to bring the meniscus in *B* and the two specks of dust in the leveling bulb in line.

A cylindrical bulb should be used, as a pear-shaped bulb is liable to lead to error from refraction. The water meniscus is now carefully read, so as to determine the total gas volume, and recorded. It is a great advantage to have the graduations on *B* run at least half-way around the tube so as to avoid parallax errors, as an error of 0.001 c.c. means an error of 0.1 c.c. CO_2 per liter. We used a thermometer lens for reading the meniscus. About 0.5 c.c. of half-normal CO_2-free NaOH was placed in *C* and allowed to run down into *B* a little at a time while tapping the apparatus.

Complete absorption of CO_2 usually took place in about 5 minutes, at 20° or 10°, but at 30° the vaseline floated out of the stopcock grease and over the surface of the alkali, and it sometimes required 30 minutes for complete absorption. Half-normal NaOH has about the same absorption coefficient for the elementary gases as the average sea-water, but it may be possible to use a stronger solution without making

gas-bubbles rise from the little sea-water above the mercury. We prefer the half-normal solution, however, because it is completely neutralized and washed out at the end of an experiment by the sea-water from the trap *T*, and it is only necessary to grease the stopcocks before another determination. In reading the gas-volume after absorption it is necessary to level the bulb, reckoning $\frac{1}{13}$ of the NaOH column as mercury.

The calculation of the results are a little complicated, but it was impossible to make a conversion table possessing great accuracy. We found the gas burette in one apparatus correct for the upturned meniscus, but it was therefore incorrect for measuring the water between the upturned and downturned menisci. A small correction in cubic millimeters given in the following table must be added to the volume of water above the mercury as measured by the scale on the gas burette *B*, depending on the total length of this scale in centimeters.

Length of 1 c.c.	Correction in c. mm.	Length of 1 c.c.	Correction in c. mm.	Length of 1 c.c.	Correction in c. mm.	Length of 1 c.c.	Correction in c. mm.	Length of 1 c.c.	Correction in c. mm.
5	24	11	11	16	8.4	21	6.7	26	5.6
6	17	12	103	17	8	22	6.4	27	5.4
7	15	13	9.7	18	7.6	23	6.2	28	5.3
8	13.5	14	9.2	19	7.3	24	6	29	5.1
9	12.5	15	8.7	20	7	25	5.8	30	5
10	11.7								

In order to make certain corrections, it is necessary to know the absorption coefficient of neutralized sea-water for CO_2. Since the half-normal HCl added to the sea-water has about the same absorption coefficient for atmospheric gases as neutralized sea-water, we assume that it does not seriously affect the absorption coefficient of the sea-water when 1 c.c. is added to 10 c.c. of the latter. Bohr's data were plotted for this purpose on the assumption that the absorption coefficient of neutralized sea-water for CO_2 is the same as that of NaCl solution of the same Cl content. The isotherms form characteristically curved lines, but in the small portion included in figure 22 the curvature was hardly perceptible and was later obliterated by the use of a straight-edge in inking-in the pencil drawing.

It is necessary to know capillary depression of the mercury in *B*, which is easily determined by opening all stopcocks and measuring the vertical distance between the mercury meniscus in *B* and the plane of the mercury surface in the leveling bulb. A mercurial barometer with glass scale is preferable, and the capillary depression must be subtracted from the reading.

In the following table for reducing the final gas-volume to 0°, 760 mm., and dryness, the temperature correction for the glass scale barometer is included. The moist gas-volume at designated temperature and

pressure is multiplied by the correction in the table. Degrees and fractions of a degree may be estimated, because 1° makes about as much difference in the correction as 4 or 5 mm. pressure.

	10°				20°				30°			
	730	740	750	760	730	740	750	760	730	740	750	760
0	.9133	.9260	.9387	.9513	.8703	.8825	.8947	.9069	.8230	.8350	.8470	.8590
1	.9146	.9273	.9400	.9526	.8715	.8837	.8959	.9081	.8242	.8362	.8482	.8602
2	.9158	.9285	.9412	.9538	.8727	.8849	.8971	.9093	.8254	.8374	.8494	.8614
3	.9171	.9298	.9425	.9551	.8740	.8862	.8984	.9106	.8266	.8386	.8506	.8626
4	.9184	.9311	.9437	.9563	.8752	.8874	.8996	.9118	.8278	.8398	.8518	.8638
5	.9197	.9324	.9450	.9576	.8764	.8886	.9008	.9130	.8290	.8410	.8530	.8650
6	.9209	.9336	.9463	.9589	.8776	.8898	.9020	.9142	.8302	.8422	.8542	.8662
7	.9222	.9349	.9475	.9601	.8788	.8910	.9032	.9154	.8314	.8434	.8554	.8674
8	.9235	.9362	.9488	.9614	.8801	.8923	.9045	.9167	.8326	.8446	.8566	.8686
9	.9247	.9374	.9500	.9626	.8812	.8935	.9057	.9179	.8338	.8458	.8578	.8698
10	.....	.....	.....	.9639	.....	.....	.....	.9191	.....	.....	.....	.8710

The mode of calculation is perhaps best shown by the following example: The analysis was made on 10 c.c. of sea-water of Cl = 19.375 per kilo, and the room was 20°, so the absorption coefficient was 0.765. The length of the graduated portion of the burette *B* was 9 cm., so the meniscus correction (Hg-H_2O and H_2O-gas) was 0.0125 c.c. and the capillary depression 3 mm. The barometer minus the capillary depression was 725 mm. The water above the mercury meniscus was 0.0565 + 0.0125 = 0.069 c.c. The total gas (0.54) minus the gas after absorption of CO_2 (0.179) equals the CO_2 (0.361 c.c.) as measured directly; but the 0.069 c.c. of water was in equilibrium with a gas, a large fraction (0.669) of which was CO_2; hence the CO_2 in that water was $0.069 \times 0.669 \times 0.765 = 0.353$. In other words, the CO_2 in the water = its volume × the CO_2 fraction + the absorption coefficient.

Practically all of this CO_2 was pumped out and then reabsorbed; hence the amount originally pumped out was 0.361 + 0.0353 = 0.3963 c.c., and this amount expanded to 39 c.c. was in equilibrium with 11 c.c. of water at the end of the first shaking. Hence this 11 c.c. finally trapped off in *T* contained $11 \times \frac{0.3963}{39} \times 0.765 = 0.0855$ c.c. of CO_2. This added to the 0.3963 c.c. brings the total up to 0.4818 c.c., and this $\times 0.8644 = 0.4165$ c.c. per 10 c.c. or 41.65 c.c. per liter at 0° and 760 mm. This correction of 0.8644 goes outside the table, but the table is probably extensive enough for most determinations, especially at a lower altitude, and the logarithm of the corrections for a more extended range may be found in the Chemiker Kalender or Landolt-Börnstein.

The apparatus was tested by making an artificial sea-water of neutral salts, distilled water that had just been boiled 15 minutes in a quartz vessel and cooled with CO_2-free air passing through it, and

some of the Na_2CO_3 used in standardizing the HCl, and weighed with standardized weights. The total CO_2 as calculated and found in this solution differed by about 0.5 per cent.

Dole used a simpler (titration) method for determining the CO_2 in sea-water, but we have not yet had time to compare it with our

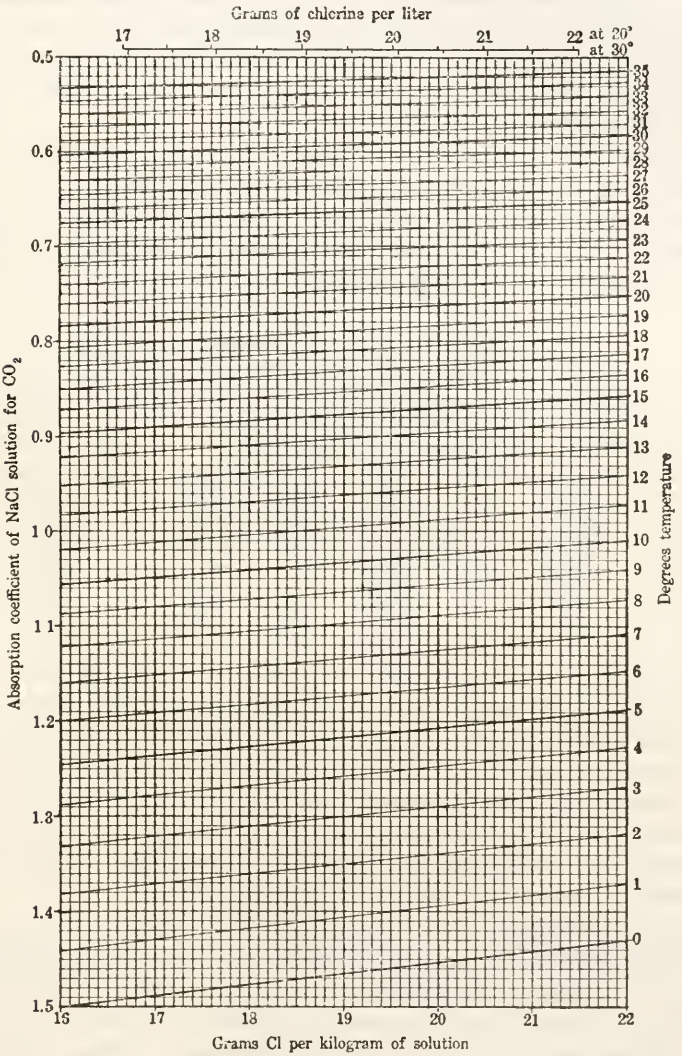


FIG. 22.—Conversion table for finding the absorption coefficient of acidified sea-water for CO_2. This table was taken from the center of a graph plotted from Bohr's data on the absorption coefficients of distilled water and solutions of NaCl for CO_2 on the assumption that the absorption coefficient of sea-water and salt solution of the same Cl content are the same, provided the sea-water does not contain alkali to combine with the CO_2. The error that may be the result of this assumption is necessarily minute and insignificant compared with errors that might arise from slightly incorrect thermometers. Each diagonal is for a different temperature. The grams of Cl per kilogram of sea-water are indicated below and grams of Cl per liter of sea-water at 20° and again at 30° is indicated above.

results. We could not use it in these experiments on account of the large sample required.

The oxygen in the sea-water may be determined on the same sample after the CO_2, by absorbing it with an alkaline solution of pyrogallol, introduced in the same manner as the NaOH solution, and the nitrogen + other inert gases may be determined by the residue. The whole calculation must be repeated for these gases, using their respective absorption coefficients, as given in the following table, calculated from Fox's data, for sea-water of Cl = 20. Fox does not state whether this is Cl per liter or per kilogram.

One of the most important requirements in such investigations as are described in this paper is accurate temperature control. All of the determinations in Minneapolis were made in a laboratory that was converted into a constant-temperature room by cutting off the steam heat and regulating the temperature by means of a nichrome wire

Degrees.	Absorption coefficient of sea-water of Cl = 20 for O_2.	Amount absorbed (c.c.) by 1 liter of sea-water saturated with air at same temperature.	Absorption coefficient of sea-water of Cl = 20 for N_2 (+argon, etc.).	Amount absorbed by 1 liter of sea-water at same temperature, shaken with air.
0	0.03825	7.97	0.0181	14.31
4	.0346	7.23	.01675	13.24
8	.03167	6.62	.0156	12.34
12	.02923	6.11	.01463	11.57
16	.02722	5.09	.01381	10.92
20	.0254	5.31	.0131	10.36
24	.02368	4.95	.01248	9.87
28	.021	4.62	.01194	9.44
30	.0213	4.46	.01168	9.24

stretched through the air in front of an electric fan and carrying 1,600 watts. The relay controlling the heating current was burned by arching because it was too small and we did not obtain a large one in time. But we found it possible to control the temperature to 0.1° by personal attention to a sensitive thermometer (graduated in tenths of a degree) placed in front of a one-sixth horse-power electric fan, and the heating current was controlled by means of a push-button.

The only time the electric heating failed to be sufficient was on two successive days when we were required to finish the experiments at 30°, although the outdoor temperature was about −30° C. It was then necessary to fill the space between the windows and dark curtains with cotton batting and to turn on the steam heat. The radiator was inclosed in a paper covering and a hole left in this of such size that the temperature did not quite reach 30°, but could be quickly raised to 30° by means of the electric regulation. It was necessary to keep a one-sixth horse-power electric fan and one or two other fans blowing on the apparatus all the time in order to keep all pieces of apparatus at the same temperature. The experiments at 10° were postponed until

the outside temperature fell to about −30° C. The experiments at 20° caused little worry in regard to temperature regulation, except on some days when the outside temperature rose to about 10°. Owing to conduction through hollow-tile walls from rooms at about 22°, it was sometimes necessary to leave the window open about an inch. Great care had to be taken then to equalize the temperature in different parts of the room. Several thermometers were compared with a standard and distributed around the room, one or two being placed on every piece of apparatus in order to detect any inequalities in temperature.

Great care was taken to prevent temperature changes in the apparatus. When necessary to handle it, this was done very gingerly, with the tips of the fingers on the extreme ends. Clamps were devised for holding the apparatus in the various necessary positions. During the night the temperature was controlled within 2° of that in the day by means of a thermostat connected with the steam-heat radiator.

HISTORICAL NOTES.

IONIZATION OF SEA-WATER.

Sea-water is a very complex solution, and it is not possible to calculate the concentrations of the different ions in it from the older analyses. Since many of these ions affect the P_H and the CO_2 content, it might be worth while to consider some general facts about the ionization. The simplest method of determining the ionization of a dilute solution of known concentration is by measuring the freezing-point. The accompanying table shows the relation of freezing-point lowering (Δ) to salinity (S) of sea-water from the determinations by H. J. Hansen (Krümmel, 1, 241). The ratio of Δ to S is 0.05424 at S = 30 and 0.05492 at S = 40, which might be interpreted to indicate that ionization increases as the concentration increases, but this is only an apparent paradox. In reality, sea-water is a concentrated solution, and it is generally true that the Δ and osmotic pressure of concentrated solutions increase more rapidly than the concentration.

S	Δ	S	Δ
30	1.627	36	1.967
31	1.683	37	2.024
32	1.740	38	2.081
33	1.797	39	2.138
34	1.853	40	2.196
35	1.910		

Electric conductivity experiments show exactly the opposite relation. Figure 23, plotted from the data of Ruppin (Krümmel, 1, 291), shows that the conductivity increases less rapidly than the concentration, and that this is almost equally true at various temperatures. Conductivity data must be considered with some caution, however, since viscosity influences conductivity. This is apparent in the change of conductivity with temperature shown in figure 24. It will be noticed that conductivity of sea-water increases very rapidly with temperature, and almost in direct proportion to it. The conductivity is doubled by a rise in temperature of 30°, and it is very improbable that ionization

increases so rapidly. Rise in temperature decreases the viscosity of water and in this way increases the conductivity. It is probable that ionization is increased slowly by dilution and also by rise in temperature of sea-water.

THE EXCESS BASE AND THE CO_2 CONTENT OF SEA-WATER.

Tornöe observed that sea-water is alkaline to litmus or rosolic acid. He added a known excess of normal H_2SO_4 to a liter of sea-water, boiled off the CO_2—and caught it in a known solution of $Ba(OH)_2$ for determining the total CO_2—and titrated the sea-water back with CO_2-

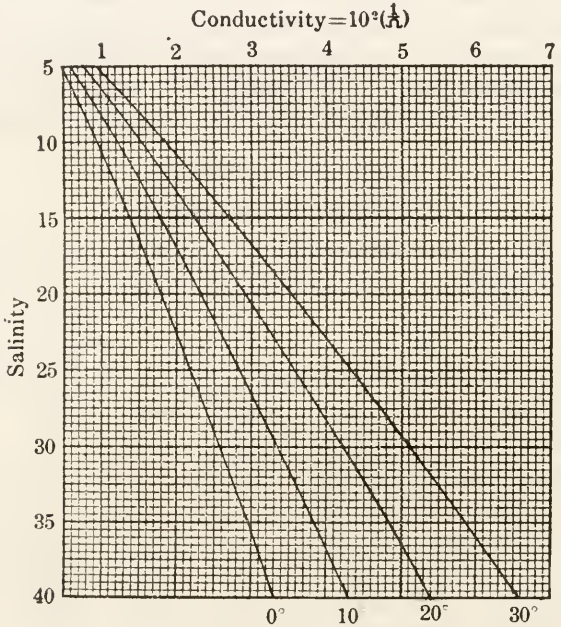


FIG. 23.—Conversion table for finding the electric conductivity of sea-water in hundredths of reciprocal ohms from the salinity and temperature, based on determinations by Ruppin. Each curve is for a different temperature.

free alkali, using phenolphthalein as indicator. He found the excess base to be 24 (in our terminology) as a mean value for the parts investigated of the North Atlantic. In titrating with phenolphthalein, the silicates and part of the borates are decomposed (*i. e.*, not re-formed when titrating back), but part of the borates and phosphates act as neutral salts, and we found that the result is about 5 per cent lower than with our method, in which boric and a small amount of phosphoric acid do not affect the result. On the other hand, the results may be too high if too great excess of acid is added or if it is boiled too long, since HCl gas is liable to pass out with the steam. It makes little difference

whether HCl or H_2SO_4 was added, since HCl is formed in sea-water from H_2SO_4 by dissociation of the large excess of chlorides.

Dittmar found that the average excess base of sea-water was about 25. In a sample of Irish Channel water he found the excess base to be 23 and total CO_2 49.7 c.c. per liter.

Tornöe found that average North Atlantic water contained 49.07 c.c. of CO_2 per liter, and Petterson found that the maximum of all of his samples contained 49.08 c.c. of CO_2 per liter (the water being drawn from a depth of 55 meters). It may be noticed that Tornöe's average is about the maximum, and probably the great majority of samples were from the depths.

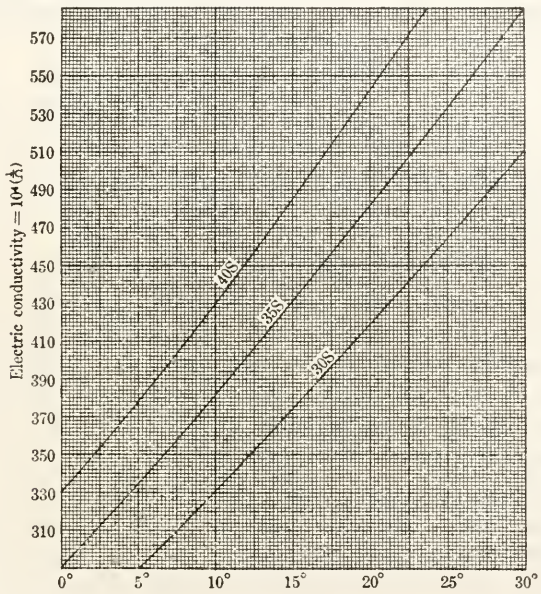


Fig. 24.—Conversion table for finding the electric conductivity in ten-thousandths of a reciprocal ohm from the salinity and temperature. Each curve is for a different salinity.

The samples collected by the *Challenger* during its cruise of three and a half years were boiled by Buchanan and the CO_2 boiled off was estimated; then they were kept several years, during which time they probably absorbed some CO_2 to replace part of that lost; then the total CO_2 they contained was determined by Dittmar and added to Buchanan's figures. Dole found about 45 c.c. of CO_2 per liter in Tortugas sea-water by a titration method.

Dittmar showed that there is an average of 0.44 per cent more lime in the water near the bottom (due to solution of shells) than at the surface of the sea, which accounts for the increase in excess base with depth. The tables of the Danish Ingolf Expedition show the following

variations in excess base near the bottom: South of Iceland and east of the barrier (Rykjanaes Ryg) excess base equals 26, west of the barrier it equals 23.7, and in Davis Strait it equals 23.5, the salinity being about 35 and bearing no relation to excess base. The surface-water of Davis Strait averaged 23.6 excess base and 34 salinity. As an exception due to special conditions, Walter and Schirlitz found that the excess base in bottom-water in depressed parts of the Bay of Naples averaged 24, whereas the surface-water averaged nearly 27. Dole found that the excess base in Tortugas water (near surface) varied from 23.7 to 25.7. Ruppin, on the *Poseidon* expedition, found that the excess base averaged 25, with 35 salinity.

Since the excess base or "alkalinity" of sea-water has been expressed in various ways, the following conversion factors are convenient for converting all results to our terminology:

Excess base = $0.8933 \times$c.c. CO_2 in normal carbonate.
= $0.4545 \times$mg. CO_2 in normal carbonate.
= $0.588 \times$mg. OH equivalent to acid used in titration.

The total CO_2 is often given in milligrams, which may be reduced to cubic centimeters by multiplying by 0.509.

CARBON-DIOXIDE TENSION OF SEA-WATER.

We express CO_2 tension in parts per 10,000. If sea-water is bottled it undergoes some fermentation with rise in CO_2 tension, unless an antiseptic is added. Krogh supposed that the addition of 1 gram $HgCl_2$ per liter caused the CO_2 tension to rise 0.35. Krogh found that the CO_2 tension of sea-water was low, except where it was diluted with fresh water from the land. Everywhere it increases with depth. In the Baltic he found tensions as high as 16 at a depth of 95 meters and salinity of 1.3. That the CO_2 tension of the sea-surface is not in equilibrium with the air is indicated by the fact that Krogh found it to vary from 1.5 to 3 in the North Atlantic. These differences were apparently partly due to change in temperature, since, if the water samples were all tested at 12.5°, the tension varied only from 2.05 to 2.95. Perhaps this water came from a uniform source (such as the Gulf Stream) with uniform tension, and the change in temperature on meeting ice and air currents diversified the tension. Since the CO_2 tension of the sea near the Gulf Stream (at Tortugas) varied from 3.3 to 4.7 (calculated from the P_H) and Krogh obtained values as low as 1.5 in the North Atlantic, we may suppose that the water maintains a relatively constant CO_2 content as it flows northward and the lowered temperature decreases the CO_2 tension. This supposition is supported by the fact that Palitzsch found that the P_H is relatively constant, and we have shown that the P_H is dependent on the CO_2 content and not on the temperature. If sea-water at 30° has 4.5 CO_2 tension, simple cooling to 0° will reduce the CO_2 tension to about 1.5.

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III.

VARIABILITY OF EGGS AND SPERM OF SEA-URCHINS.

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VARIABILITY OF EGGS AND SPERM OF SEA-URCHINS.

By A. J. Goldfarb.

INTRODUCTION.

A clear understanding of the variability in normal fresh eggs and sperm is necessary in order to appreciate and to evaluate the changes that take place in overripe germ-cells. This paper will deal exclusively with the qualitative and quantitative differences of such freshly collected sea-urchin eggs and sperm and with the differences in their early development; it will also furnish a basis for the consideration of the chemical and physical changes in aging or overripe germ-cells in later studies.

To the directors of the laboratories at Woods Hole and Tortugas I wish to extend my thanks for affording me the opportunity to carry on these researches.

TECHNIQUE.

Three different sea-urchins were used, *Toxopneustes* and *Hipponoe* at Dry Tortugas and *Arbacia* at Woods Hole.

The technique is a modification of Lillie's. As soon as possible after the urchins were collected, the eggs were removed, either by inverting each washed and partly dried individual or by removing the ovaries into separate dishes. The eggs were washed two or three times, allowed to settle by gravity in graduates, and finally enough fresh sea-water was added to make a given concentration. The dry sperm was collected from each individual separately. From this sperm any required concentration was made immediately before fertilizing the eggs. The time required to make the different suspensions varied from 30 minutes to 2 hours.

In a typical experiment 5 drops of a standard egg suspension was added to 10 c.c. of filtered sea-water and fertilized with 1 drop (0.05 c.c.) of standard sperm suspension.

The following characters were studied in some detail: (1) Size of eggs. (2) Shape of eggs. (3) Number of eggs with and without jelly layer. (4) Rate of membrane formation. (5) Number and rate of cleaving eggs.

The first three are matters of direct observation; the last two require a word of explanation, for I have found that such matters of detail are

significant in these studies. The eggs were shaken and several fields were counted, totaling 100 to 200 or more eggs. At least two counts were made, the first when most of the eggs were in the 2-cell stage—*i. e.*, 1 hour—and the second when most eggs were in the 4- or 8-cell stage—*i. e.*, 2 hours. Preliminary experiments showed that there was little or no increase in total cleavage after the second hour in *Toxopneustes* and *Arbacia*. Therefore the number of eggs that had cleaved in 2 hours was taken as the maximum. In many instances a count was made 40 minutes after fertilization, when the eggs first began to cleave. These three counts afforded the bases for computing the rate of early cleavage and the total cleavage, and also served as a check upon a possible error in the previous count.

Most of the experiments are referred to by date. Thus 7/5 means that the experiment was made on July 5, 1916.

CONCENTRATION OF THE GERM-CELLS.

It was important to determine not only whether differences in concentration of the germ-cells made for differences in membrane formation, in cleavage, etc., but also to determine what concentration gave optimum results for each species of sea-urchin. This was done in the following preliminary experiments which consisted in varying the quantity (1) of eggs of a given female, (2) of sperm, and (3) of sea-water. Tables 1 and 2 give the results of a few such experiments, with so-called "good" and "poor" eggs.

TOXOPNEUSTES.

With the volume of sea-water and the concentration of sperm constant, and with increasing numbers of eggs, a concentration was finally reached in which the rate of membrane formation, rate of cleavage, and total cleavage was considerably reduced.

In experiment 7/5 this injurious concentration was 1,084 eggs in 10 c.c. of sea-water in a Syracuse dish; 8,800 eggs in 7/8A; 9,120 eggs in 7/7A; 21,920 eggs in 7/13A; 38,400 eggs in 7/9A.

In a few experiments the injury was overcome in part by increasing the volume of sea-water 4 to 80 times; in most experiments the injury was not overcome in this manner. (See 7/7B, 7/8B, 7/13C, 7/9B.)

The decreased cleavage was due in part to insufficiency of sperm. The experiments to test this are in entire accord with those of Lillie. An increase of sperm increased cleavage 300 to 800 per cent. (See 7/8C, 7/9C, 7/13 D and E.) A decrease of sperm with low concentration of eggs did not affect the total cleavage. (See 7/13B.)

The lowered cleavage in high concentration of eggs was largely due to asphyxiation; this was evident in the experiments in which the high concentrations of eggs were gently shaken and the cleavage jumped

TABLE 1.—*Toxopneustes. Variation of membrane formation and cleavage caused by increasing the concentration of eggs and sperm.*

Experiment No.	Age in hours.		Concentration of eggs, in drops.	Cleavage.		Volume of sea-water.	No. of eggs per drop.	Fert. memb. first appeared in—	Experiment No.	Age in hours.		Concentration of eggs, in drops.	Cleavage.		Volume of sea-water.	No. of eggs per drop.
	F.	M.		Time.	P. ct.					F.	M.		Time.	P.ct.		
				min.		*c.c.*		*min.*					*min.*		*c.c.*	
7/5 A	4	½	1	84	**54**	10	17	1½	7/13 B	2	2	1	120	**92**	5	137
			2	84	**63**	10	17	1½				1	120	**92**	10	137
			4	84	**51**	10	17	1½				1	120	**95**	20	137
			8	84	**56**	10	17	1½				1	120	**98**	40	137
			16	84	**50**	10	17	1½				1	120	**97**	80	137
			32	84	**54**	10	17	1½				1	120	**91**	160	137
												1	120	**97**	320	137
7/5 B	5	1½	1	140	**76**	10	17	1	7/13 C			1	120	**81**	5	137
			16	140	**71**	10	17	1				40	120	**87**	200	137
			32	140	**75**	10	17	2				80	120	**91**	400	137
			64	140	**54**	10	17	2				160	120	**71**	950	137
			128	140	**40**	10	17	2								
			256	140	**28**	10	17	5	7/13 D			1	120	**100**	5	[5]137
												320	120	**96**	5	[3]137
7/7 A	2½	2½	1	155	**90**	10	57	....				640	120	**94**	5	[6]137
			20	155	**95**	10	57	....	7/13 E	3	3	80	120	**98**	5	[5]137
			40	155	**95**	10	57	....				160	120	**90**	5	[3]137
			80	155	**90**	10	57	....				320	120	**85**	5	[3]137
			160	155	**85**	10	57	....				640	120	**20**	5	[6]137
			320	155	**40**	10	57	....				1280	120	**3**	5	[7]137
7/7 B	3	2	40	155	**96**	20	[1]57	....	7/13 F	4	4	25	120	**40**	5	[5]137
			80	155	**64**	40	57	....				1600	120	**7**	5	[5]137
			160	155	**50**	80	57	....	7/14 A	1	1	1	120	**100**	[8]10	.....
			320	155	**12**	160	57	....				160	120	**95**	[8]10	.....
												320	120	**95**	[8]10	.....
7/8 A	2	2	1	120	**37**	10	[2]110	....				640	120	**90**	[8]10	.....
			40	120	**54**	10	[2]110	....				1280	120	**80**	[8]10	.....
			80	120	**33**	10	[2]110	....								
			160	120	**25**	10	[2]110	....	7/14 B			1	120	**100**	[9]10	.....
			320	120	**13**	10	[2]110	....				160	120	**100**	[9]10	.....
			640	120	**7**	10	[2]110	....				320	120	**95**	[9]10	.....
												640	120	**95**	[9]10	.....
7/8 B			160	120	**25**	40	110	....				1280	120	**50**	[9]10	.....
			320	120	**5**	80	110	....								
			640	120	**7**	160	110	....	7/14 C			1	120	**100**	[10]10	.....
												160	120	**95**	[10]10	.....
7/8 C			160	120	**20**	40	[3]110	....				320	120	**95**	[10]10	.....
			320	120	**44**	80	[4]110	....				640	120	**95**	[10]10	.....
												1280	120	**60**	[10]10	.....
7/13 A	2	2	1	120	**98**	10	137	....	7/14 D			1	120	**100**	[11]10	.....
			40	120	**98**	10	137	....				160	120	**95**	[11]10	.....
			80	120	**98**	10	137	....				320	120	**95**	[11]10	.....
			160	120	**94**	10	137	....				640	120	**95**	[11]10	.....
			320	120	**88**	10	137	....				1280	120	**60**	[11]10	.....
			640	120	**58**	10	137	....								

[1]Area of dishes same as above.
[2]The per cent cleavage at 120 minutes includes only normally cleaving eggs. Hence the lower figures.
[3]Used 2 drops of sperm suspension. [4]Used 4 drops of sperm suspension.
[5]Used 1 drop of sperm suspension. [6]Used 3 drops of sperm suspension.
[7]Used 6 drops of sperm suspension. [8]Shaken gently and frequently.
[9]Five drops of culture removed to 10 c.c. of sea-water, 15 minutes after fertilization.
[10]Same removed 30 minutes after fertilization. [11]Same removed 45 minutes after fertilization.

from 0 or 1 per cent to 80 per cent. In other experiments when a few drops of highly concentrated suspension were removed to 10 c.c. of sea-water, almost maximal cleavage occurred 15, 30, and 45 minutes after fertilization. (See 7/14, B, C, D.)

In *Toxopneustes*, therefore, optimum results were obtained when the egg concentration was below 8,000 in 10 c.c. of sea-water in a Syracuse dish and when fertilized by 1 drop of standard 0.05 per cent sperm suspension. In actual experimentation about one-tenth of this concentration of eggs was used, and in all experiments the sea-water was doubly filtered and stored in large quantities, to insure greater uniformity and to guard against evaporation.

HIPPONOE.

In *Hipponoe* the results were essentially similar, except that to obtain optimum results much greater concentration of sperm was necessary with a given concentration of eggs. In experiment 6/16 1 drop of 3 times standard sperm suspension fertilized only 3 per

TABLE 2.—*Hipponoe and Arbacia. Variation in cleavage caused by increasing concentration of eggs and sperm.*

Hipponoe.						Arbacia.					
Experiment No.	Concentration in drops.		Cleavage.		Volume of sea-water.	Experiment No.	Concentration in drops.		Cleavage.		Volume of sea-water.
	Eggs.	Sperm.	Time.	P. ct.			Eggs.	Sperm.	Time.	P. ct.	
			min.		*c.c.*				*min.*		*c.c.*
6/16	1	[1]1	120	**3**	5	7/28 A	1	[1]1	120	**89**	5
	1	[1]2	120	**14**	5		1	[1]$\frac{1}{4}$	120	**75**	5
	1	[1]4	120	**14**	5		1	[1]$\frac{1}{8}$	120	**66**	5
	1	[1]8	120	**20**	5		1	[1]$\frac{1}{16}$	120	**67**	5
	1	[1]16	120	**46**	5		1	[1]$\frac{1}{32}$	120	**47**	5
							1	[1]$\frac{1}{64}$	120	**35**	5
6/17	1	[1]1	180	**1**	5		1	[1]$\frac{1}{128}$	120	**0**	5
	1	[1]2	180	**14**	5						
	1	[1]4	180	**40**	5	7/28 B	1	[1]1	120	**83**	5
	1	[1]8	180	**62**	5		1	[1]$\frac{1}{16}$	120	**46**	5
	1	[1]16	180	**75**	5						
						8/9	1	[1]1	120	**45**	5
6/18	1	([2])	180	**85**	5		1	[1]$\frac{1}{16}$	120	**0**	5
	1	([3])	180	**98**	5						
	1	([4])	180	**78**	5						
	1	([5])	180	**27**	5						
	1	([6])	180	**1**	5						
	1	([7])	180	**1**	5						

[1]Standard sperm suspension.
[2]1 drop dry sperm in 2 c.c. of sea-water. Used 3 drops of this culture for fertilization.
[3]1 drop dry sperm in 4 c.c. of sea-water. Used 3 drops of this culture for fertilization.
[4]1 drop dry sperm in 8 c.c. of sea-water. Used 3 drops of this culture for fertilization.
[5]1 drop dry sperm in 16 c.c. of sea-water. Used 3 drops of this culture for fertilization.
[6]1 drop dry sperm in 32 c.c. of sea-water. Used 3 drops of this culture for fertilization.
[7]1 drop dry sperm in 64 c.c. of sea-water. Used 3 drops of this culture for fertilization.

cent of the eggs, 2 drops only 14 per cent, 8 drops 20 per cent, and 16 drops 46 per cent. The increase was roughly proportional to the increase in sperm. In 6/17B 1 drop of standard sperm suspension gave only 1 per cent cleavage, 16 drops caused 75 per cent to cleave. In 6/17A maximum cleavage occurred with 8 drops of sperm suspension; 16 drops of the same suspension made the solution milky. The matter was tested in other ways. All observations pointed to the conclusion that 15 to 80 times as much sperm was required to produce optimum results in *Hipponoe* as in *Toxopneustes*.

ARBACIA.

F. R. Lillie's careful researches with *Arbacia* have made known the optimum concentrations for this species. My observations corroborate his in every detail. Having ascertained the optimum conditions of concentration for a given surface and volume of sea-water, for each species of egg, I then undertook the study of their normal variability, which will be considered under the following headings: size and shape; jelly layer; membrane formation; cleavage.

VARIATION IN SIZE AND SHAPE OF EGGS.

In a sample lot, eggs were measured consecutively with an eye-piece micrometer (1/3 eye-piece, 1/6 objective); much care was exercised to avoid changes in size from pressure of cover-glass and to avoid errors due to poor focusing. The readings give relative values only. Repeated measurements gave so nearly the same readings that the small number of eggs cited in the table may be taken as a very close approximation to the actual situation.

Table 3 shows the extent of variation in size in the eggs of *Hipponoe*. It shows the considerable variation in the maximum, minimum, and modal size of batches of eggs from different females collected at the same time. The variation, often as great as in females collected at different times, is a result either of a general enlargement or less frequently of a shrinkage from the norm of the species. Such change in size indicates so-called "poor" eggs and is correlated with other evidences of poor physiological condition.

Similar results were obtained with *Arbacia* eggs, though the maximum mode and minimum are different.

SHAPE OF EGGS.

I have no actual counts of the variation in the shape of the eggs. Most fresh eggs are globular, though not infrequently a considerable number of the eggs are distinctly elliptical, in rare instances angular. While I have no records of the numerical relation of these three types of eggs, which intergrade, yet the presence of these three groups and the considerable variation in different females is undoubted.

VARIATIONS IN JELLY LAYER.

With India-ink solutions as used by F. Lillie, the jelly layer surrounding the egg is rendered distinctly visible and the eggs with the thick jelly coating may be readily distinguished from those with either very thin coats of jelly or no jelly. Table 3 gives the percentage of eggs with jelly envelope for each female. Counts of not less than 100 eggs were made. The number of eggs with jelly layers is usually very large: 8 out of 14 females had over 90 per cent with jelly layers; in experiment 6/28, both females had 100 per cent; in experiment 6/27 they varied from

TABLE 3.—*Variation in size and jelly of eggs of different females of Hipponoe and Arbacia.*

Date.	No.	Age of eggs.	Diameter of eggs in eye-piece micrometer readings.										Percentage of eggs with jelly.
			21	20.5	20.0	19.5	19.0	18.5	18.0	17.5	17.0	16.5	
Hipponoe.													
		hours.											
6/25	1	2	...	...	2	7	1	...	...	...	...	...	100
	2	2	...	...	...	1	9	1	...	...	...	...	63
	3	5	...	...	...	1	8	1	...	...	...	...	...
	4	0	...	...	...	4	5	...	...	...	...	...	100
6/26	3	0	...	1	3	6	3	...	...	...	...	...	86
	4	0	...	...	8	3	...	...	...	...	...	...	95
	5	0	...	...	5	10	...	...	...	...	...	...	99
	6	0	...	...	...	...	4	8	...	...	...	...	90
6/27	1	3	...	...	1	12	3	...	...	...	...	...	96
	2	3	...	...	...	11	4	...	...	...	...	...	89
	3	3	...	1	10	5	3	...	...	...	...	...	83
	4	3	2	4	3	6	...	...	...	...	...	...	80
6/28	5	½	...	...	...	1	7	4	...	...	...	...	100
	6	½	...	...	2	5	6	2	...	...	...	...	100
Total.....			2	6	34	72	54	16	0	...	...	...	
Arbacia.													
8/17	1	0	...	...	...	...	...	3	8	7	...	...	89
8/10	1	0	...	...	...	...	3	6	5	...	...	...	72
8/11	1	0	...	...	...	...	...	1	3	4	4	1	59
8/28	1	0	...	...	...	...	...	...	...	...	...	...	81

80 to 96 per cent; in experiment 6/25 one female had 100 per cent, the other as low as 63 per cent. It will later be shown that these variations in size and jelly layer in different females also serve as indices of the physiologic condition of the eggs.

Similar results were obtained for *Arbacia*. It became manifest that one must be cautious in interpreting the results, for with high concentration of sperm the percentage of jelly envelopes was often reduced. For example, in one experiment high concentration of sperm reduced the percentage of jelly by 22 per cent, 13 per cent in another, and 3 per cent in a third.

VARIATION IN MEMBRANE FORMATION.

In order to determine the normal variability in the rate of fertilization-membrane formation, it was important to ascertain the degree of variability due to differences in different samples of sperm and that due to differences in different batches of eggs. A number of the experiments to test this point are given in table 4.

TABLE 4.—*Variation in rate of membrane formation in eggs from different females.*

Date.	No. of F.	No. of M.	Time required for membranes to appear.	Percentage of cleavage in one hour.	Date.	No. of F.	No. of M.	Time required for membranes to appear.	Percentage of cleavage in one hour.
			min.					*min.*	
Toxopneustes:					*Toxopneustes:*				
7/1	1	1	6	34	7/13	1	2	[1]	65
	2	1	4	71		2	2	2½	48
	3	1	4	16		3	2	1¾	98
						4	2	1	100
	1	2	[1]	24					
	2	2	3½	24		1	3	..	61
	3	2	[1]	9		2	3	3½	48
						3	3	1⅙	85
	1	3	[1]	20		4	3	1	81
	2	3	3½	95					
	3	3	[1]	33	7/14	1	1	3½	92
						2	1	1½	47
7/9	1	5	3	15		3	1	1¾	98
	2	5	4	40					
	3	5	3	11	7/16	1	1	[1]	50
	4	5	[1]	1		2	1	[1]	74
						3	1	2½	81
7/3	1	1	[1]	80		4	1	2½	96
	1	2	[1]	23		5	1	[1]	18
	1	3	[1]	35		6	1	[1]	14
	1	4	[1]	68		7	1	2½	40
	1	5	[1]	43					
					7/19	1	1	3	82
7/2	1	1	3	..		2	1	3	96
	2	1	4	..		3	1	2	83
	3	1	3	..		4	1	1½	96
	4	1	[1]	..		5	1	1½	98
						6	1	1½	94
7/7	1	1	2	91	*Arbacia:*				
	2	1	3	41	7/28	1	1	[1]	89
	3	1	2½	67		2	1	[1]	89
	4	1	3½	86		3	1	1½	92
						4	1	2	94
7/13	1	1	2	99		5	1	1½	91
	2	1	½	83		6	1	1½	98
	3	1	1½	98		7	1	1½	97
	4	1	1½	99		8	1	1½	96

[1]No membranes appeared in first 10 minutes.

When females Nos. 1, 2, and 3 in experiment 7/1 were fertilized by male No. 1, fertilization membranes first appeared in 6, 4, and 4 minutes respectively. The same females tested at the same time by male No. 2 produced membranes in 3½ minutes in one female, and none in the other two females. Male No. 3 gave the same results as No. 2. In experiments 7/13 the membranes first appeared in 30 seconds

to $3\frac{1}{2}$ minutes. With other males fertilized at the same time, eggs from the same females formed no membrane at all. Correspondingly wide variations occurred in the other experiments (6/9, 7/3, 7/2, 7/19).

Eggs that form membranes rapidly by one male may develop them slowly by another of the same age and apparently similar condition; but usually the rate of membrane formation is conditioned by the eggs. While the rate may vary with different males, yet each female tends to keep the same relative rate—*i. e.*, there are slow-membrane-forming females and rapid-membrane-forming females.

In every experiment where different females were fertilized by the same male marked differences in the rate of membrane formation occurred. The correlation with cleavage on the one hand and jelly layers and size of the eggs on the other will be given later.

Since the rate of membrane formation varies with different males and with different females under the same experimental conditions, any comparison of results of one group of eggs with another must be made with great caution.

VARIATION IN CLEAVAGE.

Cleavage affords a definite, finely graded, and practical means of measuring variation in the eggs of different females. I have therefore given considerable attention to the variations in rate and in total cleavage in all three sea-urchins.

TOXOPNEUSTES.

The total cleavage is dependent upon the kind of male as well as the kind of female, just as we have observed in the case of the fertilization membrane. For example, 5 samples of the eggs of a given female were fertilized by 5 different males; the percentage that cleaved in 1 hour in these 5 samples was 80, 23, 35, 68, and 43, respectively, a range of 57 per cent. (See table 5, experiment 7/3.)

When different samples of eggs of a single female were fertilized by the same male the maximum difference was within 5 per cent—*i. e.*, the experimental error was below 5 per cent.

When different females were fertilized by the same male, all suspensions being standardized, the variation in cleavage was surprisingly large. It varied from 1 per cent in experiment 7/2B to 98 per cent in many experiments, 7/21, 7/19, 7/14, 7/12, etc. The range of variability as expressed by the difference between maximum and minimum cleavage for any given experiment in which a given male fertilized different females, was 11, 15, 16, 39, 48, 51, 52, 53, 54, 55, 57, 72, 74, 75, and as large as 87 per cent.

In a second series of experiments different males were used either with samples of a given female or with different females. (See experiments 7/1, 7/13.)

In 7/13, males Nos. 1, 2, 3, and 4 fertilized female No. 1 and gave 99, 83, 98, and 99 per cent cleavage respectively. With female 3, the corresponding males gave 57, 30, 50, and 46 per cent. Female No. 1 gave consistently high cleavage by all the males, and female No. 3 gave consistently low cleavage. As in rate of membrane formation, one may speak of high-cleavage eggs and low-cleavage eggs. Apparent exceptions arise when low-cleaving eggs were fertilized by high-cleavage sperm.

TABLE 5.—*Variation in percentage of cleavage, of eggs, and of sperm from different individuals of Toxopneustes.*

Date.	No. of female.	No. of male.							Age of germ-cells.	Maximum difference in cleavage percentage.
		1	2	3	4	5	6	7		
									hours.	
7/3	1	80	..	..	..	..	..	..	$\frac{1}{8}$	....
	2	23	..	..	..	..	..	..	$\frac{1}{8}$	....
	3	35	..	..	..	..	..	..	$\frac{1}{8}$	....
	4	68	..	..	..	..	..	..	$\frac{1}{8}$	....
	5	43	..	..	..	..	..	..	$\frac{1}{8}$	....
		[1]**57**	..	..	..	..	..	..	..	....
7/2	1	15	50	98	92	81	82	51	$\frac{1}{7}$	....
to	2	40	43	98	47	96	96	98	$\frac{1}{7}$	....
7/21	3	11	95	87	98	18	83	11	$\frac{1}{7}$	....
	4	1	80	..	..	14	96	..	1	....
	5	..	..	..	..	40	98	..	2	....
	6	..	..	..	..	..	94	..	1	....
		[1]**39**	**52**	**11**	**51**	**72**	**16**	**87**	..	....
7/1	1	34	24	20	..	..	..	..	1	**14**
	2	71	24	95	..	..	..	..	1	**71**
	3	16	9	33	..	..	..	..	1	**24**
		[1]**55**	**15**	**75**	..	..	..	..	..	..
7/13	1	99	83	98	99	..	..	..	$\frac{1}{2}$	**16**
	2	25	64	93	100	..	..	..	$\frac{1}{2}$	**75**
	3	57	30	50	46	..	..	..	$\frac{1}{2}$	**27**
		[1]**74**	**53**	**48**	**54**	..	..	..	..	..

[1]Maximum difference.

Since the variability in the reactions between these fresh eggs and sperm is so amazingly large, one should be cautious about comparisons of fertilized eggs from different individual sea-urchins, even when of one and the same species; yet this error is commonly made.

TABLE 6.—*Variation in cleavage of germ-cells of different individuals—Hipponoe.*

Date.	No. of female.	No. of male.	Age of germ-cells.		Per cent cleavage.	Maximum difference in cleavage percentage.	Time since fertilization.
			Female.	Male.			
			hours.	*hours.*			*hours.*
6/21	1	1	0	0	45	....	3
	1	2			76	**31**	
6/23	1	1	0	0	16	....	2
	2	1			6	**10**	
6/27	1	1	0	0	91	....	1½
	2	1			12	....	
	3	1			85	....	
	4	1			8	**83**	
6/26	1	1	0	0	83	....	2
	2	1			66	**17**	
7/1	1	1	0	0	90	....	1½
	2	1			54	....	
	3	1			59	....	
	4	1			47	....	
	5	1			16	**94**	
7/2	1	1	0	0	56	....	1½
	2	1			51	....	
	3	1			74	....	
	4	1			57	....	
	5	1			70	....	
	6	1			27	**47**	
7/6	1	1	0	0	10	....	1½
	2	1			37	....	
	3	1			19	....	
	4	1			64	**54**	
7/16	1	1	0	0	0	....	2
	1	2			0	....	
	1	3			0	....	
	3	4			44	....	
	3	5			0	....	
	3	6			8	**44**	
6/29	1	1	0	0	19	....	2½
	2	1			94	....	
	3	1			73	**75**	
6/29	4	5	1⅔	1⅔	92	....	1½
	5	5			97	....	
	6	5			83	**14**	
7/8	1	1	0	0	52	....	1⅔
	2	1			65	....	
	3	1			58	....	
	4	1			41	**24**	
7/8	5	1	2¼	2¼	92	....	1½
	6	1			22	....	
	7	1			38	....	
	8	1			77	**70**	
7/3	1	1	0	3	62	....	2
	2	1			94	....	
	3	1			13	**81**	
6/28	1	1	0	7	24	....	1½
	1	2			57	**33**	
7/30	1	1	½	½	71	....	1½
	2	1			76	**5**	

HIPPONOE.

The variation in cleavage of *Hipponoe* eggs was essentially the same as in *Toxopneustes*. The data are brought together in table 6.

Samples of eggs of a given female fertilized by different males gave 45 and 76 per cent cleavage, respectively, a range of 31 per cent. (Experiment 6/21.)

Eggs of different females fertilized by the same male varied from 5 to 81 per cent in the 13 experiments cited in table 6. The maximal difference in each experiment was 5, 9, 10, 17, 24, 47, 54 per cent and as large as 70, 74, 75, and even 81 and 83 per cent.

In these experiments so called "good," "poor," and "bad" eggs were used. These terms will later be defined in definite chemico-physical entities.

TABLE 7.—*Variation in cleavage of eggs and sperm of different Arbacia individuals.*

Experiment No.	Age of germ-cells.		No. of male.	Egg No. 1.	Egg No. 2.	Egg No. 3.	Egg No. 4.	Egg No. 5.	Egg No. 6.	Egg No. 7.	Egg No. 8.	Maximum difference (per cent).
	Female.	Male.										
8/12.....	4¼	4¼	1	81	58	1	77	91	..	..	..	**90**
	4½	4½	2	72	69	4	62	53	..	..	..	**68**
8/13.....	23	23	1	75	70	3	0	74	..	..	..	**75**
8/14.....	1½	1½	1	80	74	99	96	100	..	..	..	**26**
	5	5	5	93	90	100	85	95	..	..	..	**15**
8/16.....	1½	1½	1	100	98	100	88	98	92	100	..	**12**
7/28 (1 hour)...	0	0	1	83	89	92	94	91	98	97	96	**15**
7/28 (2 hours)..	0	0	1	92	98	97	97	98	97	99	99	**7**
8/2.....	0	0	1	100	100	..	..	..	..	..	..	**0**

ARBACIA.

A few observations were made upon *Arbacia*, which live in much deeper and colder waters. The results are shown in table 7.

In experiment 8/2, table 7, eggs of different females fertilized by the one male gave 100 per cent cleavage for each. In experiment 7/28 all 8 females gave a high cleavage; the maximum difference was 15 per cent at end of 1 hour and only 7 per cent at end of 2 hours. In the other experiments the range of variability was much greater—12, 15, 26, 68, 75, and 90 per cent in different experiments. There were low-cleaving females (see experiments 8/12 and 8/13); others were sterile or practically so (see 8/12 and 8/13); others high-cleaving females. The eggs appeared alike, yet upon actual test they showed a very marked range of variability. On some days all the females seemed to be high-cleaving females, as in 7/28; on other days there was marked variation, as in 8/12, 8/13, etc.

CORRELATION OF THE DIFFERENT VARIATIONS.

These observations and experiments furnished unmistakable evidence of a high correlation of these variants in all three sea-urchins. From table 3 the size of eggs and the percentage of eggs with jelly envelopes may be compared; also in table 4 the rate of membrane formation and total cleavage. From the different experiments it was observed that the following characters and characteristics were closely correlated, namely:

No. 1. Globular shape of all or nearly all of the eggs.
No. 2. Large percentage of the eggs with the jelly envelope.
No. 3. Size of the eggs close to mode, with very little variation.
No. 4. (*a*) Fertilization membrane formed in about 2 minutes.
(*b*) Large percentage of the eggs form a membrane.
No. 5. (*a*) A rapid rate of first cleavage.
(*b*) A high percentage of cleavage.

Vice versa, the following were closely correlated:

No. 1. A *large* percentage of *elliptical* eggs.
No. 2. A *low* percentage of eggs with their *jelly* envelopes.
No. 3. Eggs usually larger than mode, and much variation.
No. 4. (*a*) Fertilization membranes slow to appear.
(*b*) Small percentage of eggs form membranes.
No. 5. (*a*) Slow rate of first cleavage.
(*b*) Low percentage of cleaving eggs.

DISCUSSION.

An adequate understanding of these variations can not be had without a knowledge of the results of experiments with aging germ-cells. It is expected that such results will appear in a later publication, but at present I wish merely to point out the nature of some of the variants and to call attention to the marked range of differences in each of the variants in *fresh germ-cells*. If these facts are thoroughly appreciated, not only will many conflicting results on the physiology of eggs and sperm disappear, but a clearer understanding of the situation may result.

A number of investigators have remarked on the fact that under apparently identical conditions varying experimental results were obtained, and have suggested that the differences were due to some physiologic difference in the eggs.

Stockard (7) stated "the fact that a number of eggs when subjected to the same solutions do not all respond in a like manner" was due to "*differences in individual resistance and vigor.*"[1]

F. R. Lillie (2) noted that "the failure to obtain exactly the same curve * * * was due in part to the *natural variability of different lots* of eggs and sperm." Elsewhere he asserts that the percentage of cleavage was more *dependent on the conditions of the sperm* than upon its concentration.

[1]All italics are mine.

J. Loeb and H. Wasteneys (5) believed that the different results on the effects of weak and strong bases on the oxidation of eggs of the sea-urchin may be due to *differences in the eggs.*

Wasteneys (8) later, in working upon the oxidation of sea-urchin eggs, concluded that the variations in his results were due to *differences in sensitiveness of eggs of different females* and perhaps to differences of temperature.

R. S. Lillie (3), from a study of the rate of swelling of eggs in diluted sea-water, also concluded that the variability in rate was due to the *condition of the eggs.*

In studying the fertilizin phenomenon, F. R. Lillie (1) arrived at a more adequate appreciation of the nature of this variability when he held that the *condition of the eggs*, whether ripe or immature, fresh or stale, with or without jelly, is more important than concentration of the eggs. Elsewhere (p. 568) he remarked that "*the condition of the gonads* is the most variable thing in summer sea-urchins."

R. S. Lillie (3) made a notable contribution when he concluded that resistance to osmotic disruption in dilute sea-water was "a convenient index of the physiologic condition of the plasma membrane" and that "an intimate connection exists between the general physiologic condition of the egg and the physiologic state of the plasma membrane."

We are not justified in assuming that all ripe eggs freshly removed from the bodies of the sea-urchins are in a uniform or nearly uniform physiologic condition or that at a subsequent interval in sea-water the eggs continue to remain in a fairly uniform physiologic condition.

This study and later ones have forced upon me the conclusion that these assumptions are without basis. Eggs freshly removed from the body are *not in the same physiologic condition*, as measured by any of the tests I have proposed. Given an originally limited variability of the eggs—for the chemico-physical explanation of which see Loeb and Chamberlain (4)—which variability I shall term the primary variability, further variations and types of variants are superimposed by reason of the fact that the eggs do not all ripen[1] at the same time within the body. Further variability is due to the fact that the eggs are subjected to the injurious influences of the body fluid for intervals varying with the time since maturation.

When such eggs are placed in sea-water the physiologically different groups of eggs are again affected very unequally.

The totality of these varying influences produces marked variations of a number of characters among the eggs of a given female, as well as the eggs of different females.

We have no direct or simple measure of the time since maturation of the egg, nor of the physiological condition of the eggs. But one can

[1]Eggs are ripe in this sense at the moment when germinal vesicle breaks down.

obtain a very definite idea of the physiological condition of any sample of eggs by ascertaining the extent of certain changes in size, shape, dissolution of jelly layer, retardation of rate of membrane formation, and rate of cleavage and total cleavage, all of which, and possibly others, serve as different and corroborative indices of their physiological condition. The nature of the chemico-physical factors involved in these variations was made known from a study of aging eggs.

From this it follows that for a study of the physiology of the germ-cells, or for any experimental work that involves a comparison of germ-cells from different individuals, it is not sufficient to use eggs of the same chronologic age, but by actual tests, as shown above, to choose eggs of the same physiologic age—*i. e.*, eggs most nearly in the same physiologic condition. If this be done we should expect less conflicting results than have obtained heretofore.

CONCLUSIONS.

1. The optimum conditions of temperature, volume of sea-water, surface exposure, and concentration of eggs and of sperm were ascertained.

2. The eggs and sperm used in these experiments *were immediately removed from freshly collected sea-urchins.*

3. Three species of sea-urchins were used, *Toxopneustes* and *Hipponoe* of shallow tropical waters and *Arbacia* of deeper, colder waters. The fundamental results for all these were in entire accord.

4. The *Arbacia* germ-cells are less variable than either of the tropical forms.

5. Variability, with respect to cleavage, differs with different individuals. The eggs of one female when fertilized by a given male may give markedly different percentages of cleaving eggs when fertilized by a second male. The difference is usually much greater, all other conditions remaining constant, when eggs of different females are fertilized by the same male. The relative difference is usually constant. Some females have a high percentage of cleaving eggs; other females a low percentage. Cleavage is a function of the kind of eggs used.

6. The variations studied were: size and shape of eggs; presence of jelly envelope; membrane formation; cleavage.

7. There is a surprising range of variability for each of these characters and characteristics, less for size or shape, greater for jelly and membrane formation, and greatest for cleavage.

8. The eggs of some females showed little variation in size from the norm, were usually globular, and a large proportion had the characteristic jelly envelope. These eggs were in good physiologic condition.

9. The eggs of other females varied considerably in size from the norm, had a high percentage of elliptical eggs and a low percentage of intact jelly envelopes. These eggs were in poor physiologic condition.

10. In some females the eggs formed fertilization membranes in about 2 minutes, others required 3 to 6 minutes, or formed no membranes at all. Rapid membrane formation was associated with small variability of size, globular shape, and high jelly count; slow formation with enlargement or considerable variation in size, high percentage of elliptical eggs and low jelly count. The former were in good, the latter in poor physiologic condition.

11. The rate of early cleavage and the total cleavage varied most widely. The total cleavage varied from complete sterility to 100 per cent cleavage. High cleavage is correlated with normal size, globular shape, large jelly count, and rapid membrane formation; and *vice versa*, low cleavage with the opposite conditions.

12. This amazingly large variation is due to:

a. A primary, small variability in ripe eggs.
b. The effect of chemico-physical agencies acting on the ripe eggs within the body of the female.
c. A variation in time or ripening of different groups of eggs within a given female.
d. The time between maturation and removal of the eggs to sea-water, which time may be different for different females.
e. The differential effect of sea-water upon physiologically different eggs.

Eggs which have been removed from different individuals at the same time and are of the same chronologic age are not necessarily in the same physiologic condition. The extent of the change may be determined by the study of their variations.

13. For certain experimental work the eggs should be grouped, not according to age—*i. e.*, the time since removal from the body—but according to their physiologic state, which may be accurately determined by the various tests suggested above.

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IV.

ANALYTICAL SEARCH FOR METALS IN TORTUGAS MARINE ORGANISMS.

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ANALYTICAL SEARCH FOR METALS IN TORTUGAS MARINE ORGANISMS.

By Alexander H. Phillips.

INTRODUCTION.

The problem upon which it is hoped that these analyses will cast some light was stated in Year Book No. 14, page 193, of the Carnegie Institution of Washington. A large number of specimens were collected and, while only a few of them have been analyzed for metals, the results justify the present publication, leaving the discussion to some future time when the analyses of all the samples or specimens collected, as well as others from various localities, might be included.

The specimens were dried in the air-bath at 110° C., were ground, and bottled in bottles with tight-fitting stoppers. Some specimens slowly decomposed at 110° C. and constantly lost weight; in such cases the specimens were heated in the bath for 8 hours and then bottled as if dry. The metals determined were iron, manganese, zinc, copper, and lead. For the determination of zinc, copper, and lead, when the dried material was sufficient, 20 grams were used as a sample; when it was not possible, through lack of material, to use 20 grams, the results, as tabulated for convenience of comparison, are all calculated to 20 grams.

METHOD.

The method of analysis followed is that advised by the U. S. Department of Agriculture, Bureau of Chemistry, Bulletin No. 107 (revised), with slight modifications, as tin was not sought. The sample was incinerated in a muffle at a low red heat, not high enough to fuse the chlorides. After solution of the ash in HCl and evaporation to dryness, sulphides were precipitated in a hydrochloric-acid solution. They were then dissolved in HNO_3, when copper and lead, if any, were determined electrolytically in the nitric-acid solution. Iron and phosphoric acid were separated from zinc in the filtrate from the sulphides by the basic-acetate method, when the zinc was precipitated as a sulphide in an acetic-acid solution and weighed as ZnO. If this zinc oxide was found to be impure it was dissolved in hydrochloric acid, filtered, and the filtrate was boiled with an excess of NaOH in platinum,

any precipitate filtered off, and the zinc reprecipitated with Na_2CO_3 and again weighed as ZnO.

Iron was estimated in a separate sample, usually 2 grams, by titration with permanganate. Manganese was also estimated in a separate sample, by the colorimetric method, and both, as tabulated below, are calculated to amounts contained in 20 grams of the dry sample. The weights, etc., are given in grams.

Specimen No.	Sample taken for analysis	Weight complete.	Weight of shell.	Weight of tissues.	Weight of liver.
614 B C 1....	20 grams of liver.....	4,365	2,990	1,375	157
614 B C 2....	20 grams of liver.....	3,705	2,595	1,110	105
614 K 1.....	20 grams of liver.....	4,265	2,875	1,390	405
614 K 2.....	20 grams of liver.....	3,730	2,485	1,245	315
614 R.....	20 grams of tissues.....	770	610	160	...
614 A T.....	20 grams of liver.....	21,015	17,835	3,180	585
614 A U.....	5 grams of liver.....	1,765	1,527	238	57
614 D 2.....	20 grams of tissues.....	2,925	2,555	370	...
614 E F G....	20 grams of tissues.....	8,368	7,046	1,322	...
614 L.....	20 grams of tissues.....	1,225	787	438	...
614 A S.....	10 grams.....	1,515	.....	.....	[1]201
614 A S 1....	2 grams of liver.....	1,515	.....	.....	22
614 A S 2....	20 grams of liver.....	.....	.....	.....	67
615 E.....	20 grams.....	.....	.....	.....	1,035
615 E 2a.....	10 grams[1].....	.....	.....	[1]190	.....
614 C B 1....	15 grams.....	450	.....	.....	.....
614 C B 2....	20 grams.....	.....	.....	.....	.....
614 C B 3....	3 grams.....	.....	.....	.....	18.5
614 A 1.....	20 grams.....	2,670	.....	1,945	.....
614 A 2.....	20 grams.....	2,670	.....	725	.....
614 N.....	20 grams.....	2,215	.....	.....	.....
614 B Q.....	20 grams.....	395	.....	.....	.....

Specimen No.	Weight dried at 110° C.	Copper.	ZnO	Fe	MnO	PbO_2
614 B C 1....	[2]69.2	0.0434	0.0036	0.0121	0.00022	0.00005
614 B C 2....	[2]45.1	.0745	.0123	.0208	.00020	.00005
614 K 1.....	[2]180.8	.0007	.0019	.0108	.00016	.....
614 K 2.....	[2]136	.0019	.0014	.0081	.00008	.....
614 R.....	41.5	.0006	.0014	.0112	.0011	.....
614 A T.....	[2]175.9	.0006	.0019	.0176	.00355	.....
614 A U.....	[2]8.1	.0008	.0076	.0397	.0043	.....
614 D 2.....	81.5	.0025	.0051	.0193	.0021	.....
614 E F G....	322	.0002	.0038	.0073	.0022	.....
614 L.....	101	.0007	.0077	.0114	.00016	.....
614 A S.....	[1]17.6	.0140	None.	.0175	.0014	.....
614 A S 1....	4	.0180	.0040	.0057	.000016	.....
614 A S 2....	31.7	.0222	.0038	.0056	Trace	.....
615 E.....	219	.0034	.0133	.0054	.0001	.....
615 E 2a.....	17.24	.0170	.0014	.0057	.00084	.....
614 C B 1....	22.4	.00027	.0011	.0079	.0002	.....
614 C B 2....	92	.0003	.0016	.0087	.0002	.....
614 C B 3....	5.4	.0022	.0020	.0124	Trace	.....
614 A 1.....	404	Trace	.0043	.0057	Trace	.....
614 A 2.....	74	.0004	.0013	.0002	Trace	.....
614 N.....	215	.0003	.0006	.0235	.0002	.....
614 B Q.....	44	.0004	.0032	.0229	.0002	.....

[1]Blood. [2]Liver.

DESCRIPTIONS OF SPECIMENS ANALYZED.

614 B C 1. *Fasciolaria gigantea;* horse conch; liver of an immature male.
614 B C 2. *Fasciolaria gigantea;* liver of a much older individual, sex not noted.
614 K 1. *Cassis* (?); queen conch; liver only; sex not noted.
614 K 2. *Cassis* (?); queen conch; liver only; sex not noted.
614 R. *Strombus bituberculatus;* all soft parts of 6 females are represented in the samples analyzed.
614 A T. *Strombus gigas;* represents the livers of 8 individuals of both sexes.
614 A U. *Strombus gigas;* the liver of a much younger individual than any included in 614 A T.
614 D 2. *Strombus gigas;* represents all the soft parts of a single individual with a very old, battered, and thick-lipped shell.
614 E F G. *Strombus gigas;* represents all the soft parts of 3 individuals, all in the first year of maturity.
614 L. *Fulgur perversus;* represents all the soft parts of 4 adult individuals.
614 A S. *Palinurus;* crayfish; represents the blood only.
614 A S 1. *Palinurus;* crayfish; liver of a single individual.
614 A S 2. *Palinurus;* crayfish; liver of a much larger individual.
615 E 1. *Limulus polyphemus;* livers, ovaries, and testes of four individuals, two males and two females, collected at the Marquesas.
615 E 2a. *Limulus polyphemus;* 190 c.c. of blood.
614 C B 1. *Aplysia;* sea cat; one complete individual.
614 C B 2. *Aplysia;* sea cat; five complete individuals.
614 C B 3. *Aplysia;* sea cat; the livers of two individuals.
614 A 1. *Holothuria bermudiana;* muscular tissue, etc., of several large individuals, less the intestines.
614 A 2. *Holothuria bermudiana;* intestines of the individuals in 614 A 1.
614 N. *Ciona atra;* black tunicate, living on the walls of the moat of Fort Jefferson.
614 B Q. A gray tunicate also from the moat of Fort Jefferson.

The constant occurrence of both copper and iron in the analyses is to be expected, but the large amounts of copper in some and its variability, even in individuals of the same species, is to be noted. Zinc is present in all the specimens analyzed except the crayfish blood, and as the sample was exhausted in the first analysis this could not be tested further. Since the Tortugas Islands are far removed from any possible contamination of the sea-water, zinc must be considered as a normal constituent of these forms. Manganese is present in all the specimens analyzed, but varies considerably. The most remarkable occurrence, however, is that of lead in the liver of *Fasciolaria gigantea* in quantity just sufficient to weigh, in a 20-gram sample of dried liver, but quite enough to yield good qualitative tests.

V.

ON THE COMPOSITION OF CASSIOPEA XAMACHANA AND THE CHANGES IN IT AFTER STARVATION.

By S. HATAI,

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One figure.

ON THE COMPOSITION OF CASSIOPEA XAMACHANA AND THE CHANGES IN IT AFTER STARVATION.

By S. Hatai.

INTRODUCTION.

Mayer (1914) reported that in *Cassiopea* the percentage of nitrogen to the total solids remains constant during the entire period of starvation; he infers that "no appreciable chemical change occurs in the composition of its body, and that there is no appreciable selective use of different substances at different times during the progress of starvation." This is remarkable, since the starving mammalian body reveals a totally different relation, owing to the rapid disappearance of reserve substances, such as carbohydrates and fats, during the earlier period of starvation, followed by a slow consumption of protein substances later. Thus the starving mammalian body gives different percentage values for the nitrogen at different periods of starvation, especially in the earlier stages.

The general anatomy of *Cassiopea* suggests that since by far the greater fraction of the body is represented by the reserve jelly-like substance, while the amount of living cellular elements is small, constancy in the percentage of nitrogen means that practically all the nitrogen is represented by that of the jelly-like substance, which by its relative abundance masks those chemical changes that may occur within the epithelial elements. To test this point the experiments to be described were undertaken.

NORMAL GROWTH OF DIFFERENT PARTS OF THE BODY.

Cassiopea may be divided into three distinct parts: (1) Mouth-organs, (2) umbrella, and (3) velar lobes, which differ not only morphologically, but also in their absolute weights and in the relative amount of cellular and non-cellular constituents. It was, therefore, thought desirable to study the normal growth of these parts in order to determine whether the starving cassiopea loses weight uniformly or whether the loss is dissimilar in the three parts concerned. Altogether 37 normal examples of *Cassiopea* of various body-weights were examined, and the results obtained are given in table 1.

The individual records were plotted, together with the average values, with a view to showing at a glance the extent of variation, and are given in figure 1.

Table 1 shows that the three parts increase in weight with the increase of the entire body-weight, but the relative weights of these

parts change as the body becomes heavier. The greatest increase in relative weight is made by the velar lobes, while those made by the mouth-organs and the umbrella follow in the order named. It should be noted that, although the mouth-organs show the greatest weight during most of the animal's life, yet in the earlier period, where the entire body weighs less than 0.5 gram, the umbrella has a greater weight than the mouth-organs.

TABLE 1.—*Different body-weights of the normal Cassiopea, the weights of mouth-organs, umbrella, and velar lobes.*

Entire body.	Mouth-organs.	Umbrella.	Velar lobes.	No. of medusæ.
grams.	*grams.*	*grams.*	*grams.*	
0.098	0.040	0.056	0.002	1
.316	.141	.154	.022	5
.806	.384	.359	.063	4
8.990	5.918	2.550	.521	5
54.219	32.182	18.103	3.933	7
118.313	66.800	42.092	9.421	6
163.993	92.694	59.080	12.219	5
234.284	140.497	78.255	15.531	4

The graphs in figure 1 show that the growth in weight of these three parts on body-weight is almost linear. It would be interesting to determine how the growth curve of *Cassiopea* with respect to age appears, but for the necessary data we must await future investigations.

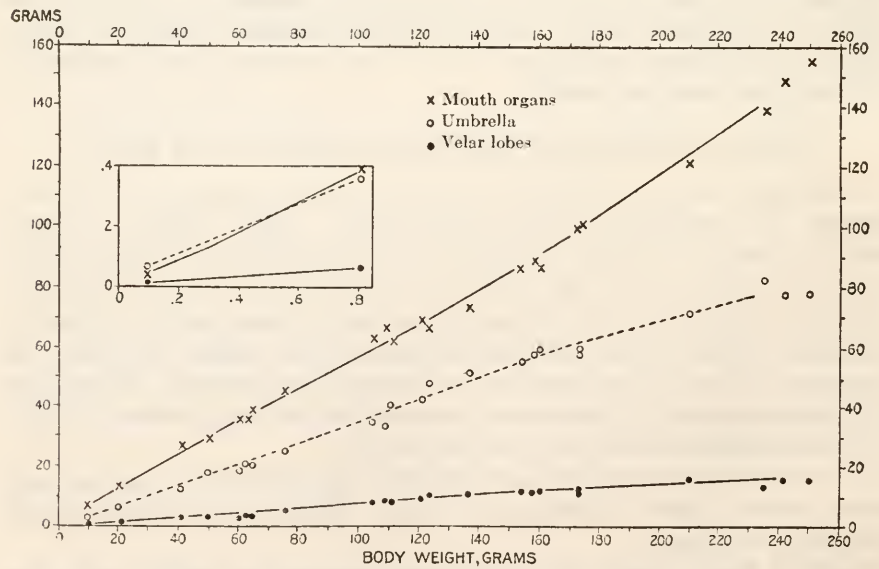

FIG. 1.—Showing the growth of three parts of the body according to the increasing weight of *Cassiopea xamachana*.

ANALYSIS OF ENTIRE BODY.

Several freshly caught cassiopeas were weighed and then placed in the oven for one week at a temperature of 90° to 95° C. The percentages of water and of solids were thus determined and the solids again were analyzed for nitrogen and ash. The results of these determinations are given in table 2.

TABLE 2.—*Fresh weight, dry weight, water, nitrogen, and ash in a series of Cassiopeas of increasing body-weights.*

Body-weight, fresh.	Body-weight, dry.	Water.	Solids.	Total nitrogen.	Nitrogen in solids.	Total ash.	Ash in solids.	Remarks.
		p. ct.	*p. ct.*	*mgm.*	*p. ct.*	*mgm*	*p. ct.*	
0.402	0.023	94.28	5.72	.....	.....	...	.....	
1.000	.055	94.50	5.50	.....	.....	...	.....	
1.383	.085	93.86	6.14	2.77	3.226	...	.....	Average of 3.
1.540	.085	94.48	5.52	.....	.....	...	.....	
1.786	.113	93.68	6.32	3.83	3.402	...	.....	Average of 3.
5.559	.329	94.08	5.92	9.80	2.983	...	.....	Average of 2.
5.876	.333	94.33	5.67	.....	.....	174	52.16	
7.496	.454	93.95	6.05	14.56	3.207	...	.....	
8.257	.483	94.15	5.85	13.86	2.870	...	.....	
9.128	.540	94.08	5.92	.....	.....	285	52.78	
10.060	.592	94.12	5.88	17.78	3.003	...	.....	
10.931	.625	94.28	5.72	17.40	2.784	...	.....	
10.958	.650	94.07	5.93	.....	.....	329	50.62	
20.530	1.194	94.19	5.81	27.16	2.275	...	.....	
		[1]94.14						
100.055	5.401	95.10	4.9	138.59	2.566	...	.....	Mayer, 1914.
109.641	5.43	95.10	4.9	116.58	2.147	...	.....	Mayer, 1914.

[1]Average.

PERCENTAGE OF WATER.

It is interesting to note that the percentage of water is practically identical in all sizes of *Cassiopea* here examined, thus indicating that the jelly-like substance of which the body of *Cassiopea* is largely composed remains nearly unmodified throughout the life cycle. This is in remarkable contrast to the water-content of mammalian bodies at various ages in which the younger individuals give the higher percentage of water and *vice versa* (Hatai, 1916).

PERCENTAGE OF NITROGEN IN TOTAL SOLIDS.

The nitrogen in the total solids was determined by the usual Kjeldahl method and the results are given in table 2. We notice that despite the similarity of the water-content, the percentage of nitrogen in the solids shows a progressive decrease with increasing body-weight. Although this difference in the relative nitrogen-content between the smaller and larger animals is not very large, nevertheless the existence

of such a difference can not be doubted. We assume that this difference in the nitrogen-content is due to the relative abundance of cellular elements in the smaller individuals, contrasted with an excess of the reserve materials composed of organic substances poorer in nitrogen in the larger individuals. We thus find that, within the limits taken, the reserve jelly-like substance and cellular elements probably both have the same water-content, since otherwise the same percentage of water throughout the series could not be expected.

PERCENTAGE OF ASH IN SOLIDS.

Since the percentage of water is similar in all the sizes of *Cassiopea* examined, and since both cellular elements and reserve materials have a like water-content, we should anticipate that the ash-content would be also similar in the several stages, and the determination of the ash-content on three individuals supports this conclusion. The proportion of ash is as high as 52 per cent, thus making the organic substances less than half of the entire solids.

ANALYSIS OF DIFFERENT DIVISIONS OF THE BODY.

Using four cassiopeas, which also appear in table 2, the three different parts of the body were separately analyzed. The results are shown in table 3.

TABLE 3.—*Water-content, solids, and nitrogen in three different parts of the normal Cassiopea.*

Parts.	Weight, fresh.	Weight, dry.	Water.	Solids.	Total nitrogen.	Nitrogen in solids.
	grams.	*grams.*	*p. ct.*	*p. ct.*	*mgm.*	*p. ct.*
Mouth-organs....	4.925	0.311	93.69	6.31	10.50	3.376
Umbrella.....	2.133	.118	94.47	5.53	2.66	2.254
Velar lobes.....	.438	.025	94.29	5.71	1.40	5.600
	7.496	.454	93.94	6.06	14.56	3.207
Mouth-organs....	5.553	.334	93.99	6.01	9.94	2.976
Umbrella.....	2.282	.123	94.61	5.39	2.52	2.048
Velar lobes.....	.422	.026	93.84	6.16	1.40	5.384
	8.257	.483	94.15	5.85	13.86	2.870
Mouth-organs....	6.510	.399	93.87	6.13	12.18	3.052
Umbrella.....	2.906	.153	94.74	5.26	3.64	2.379
Velar lobes.....	.644	.040	93.79	6.21	1.96	4.900
	10.060	.592	94.11	5.89	17.78	3.003
Mouth-organs....	13.116	.795	93.94	6.06	18.90	2.390
Umbrella.....	6.283	.329	94.76	5.24	5.74	1.745
Velar lobes.....	1.131	.070	93.81	6.19	2.52	3.600
	20.530	1.194	94.18	5.82	27.16	2.275

PERCENTAGE OF WATER.

It will be seen that the percentage of water in these three different parts of the body is nearly the same. The water-content of the umbrella is, however, constantly higher by about 0.7 per cent as contrasted with the two remaining parts. This is interpreted as probably due to the inclusion in the umbrella of the contents of the stomach, which are mainly liquid. The fact that the velar lobes (which have a large proportion of cellular elements) and the mouth-organs (which have a large proportion of reserve jelly-like substance) are practically identical in water-content shows not only the same degree of water inhibition in both cells and reserve substance, but also an equal distribution of salts.

PERCENTAGE OF NITROGEN IN THE SOLIDS.

Despite the fact of the uniform distribution of water and probably of salts in the body, the nitrogen-contents of these three parts are different. Table 3 shows that the nitrogen-content is highest in the velar lobes, where the cellular elements are most abundant, while it decreases in the mouth-organs and umbrella in the order named. The higher nitrogen-content in the mouth-organs, as compared with the umbrella, is assumed to be due to the relatively greater abundance of the cellular elements in the former. We can infer from these data that the ground substance, or the reserve jelly-like substance, must be composed of organic materials poor in nitrogen. I shall discuss this point later in connection with the probable chemical nature of the reserve material.

The observations made on the normal cassiopea may be summarized as follows:

(1) Relative weights of mouth-organs, umbrella, and velar lobes differ somewhat according to the size of the entire body.
(2) The water-content of the entire body, as well as of different parts, is practically identical throughout the animal's life cycle, so far as followed.
(3) The percentage of nitrogen in the solids is highest in the smallest medusa and the values decrease progressively with increasing body-weight. The percentage of nitrogen is highest in the velar lobes and decreases in the mouth-organs and umbrella in the order named. All these observations are interpreted as indicating that in the structures in which the cellular elements are abundant the nitrogen-content tends to be high.
(4) The ash-content, like the water-content, is probably the same not only in the cassiopea as a whole, but also in each of the three parts throughout the life cycle.

CHEMICAL NATURE OF THE GELATINOUS SUBSTANCE OF CASSIOPEA.

The chemical nature of the jelly-like substance (reserve substance) of various forms of medusæ has been examined by several investigators, but so far as I am aware nothing has been definitely established as to the chemical nature of this puzzling structure.

Krukenberg (1882) considers this substance to be composed of protein material which is easily digested with either pepsin or trypsin and splits off leucin, tyrosin, and other substances not identified. Very recently Macallum (1903) published his extensive investigations on the inorganic constituents of the medusæ *Aurelia* and *Cyanea*, and mentions in the text that "the jelly is constituted of an almost infinitesimally minute network formed of a proteid." In another place Macallum states that "it must be noted that the total amount of proteid in *Aurelia* is very small, ranging between one-seventh and one-eighth of 1 per cent of the total weight of the organism," but in no place in the paper does Macallum mention either the kind of protein present or give any analytical data to show how he determined the quantity of this protein. There are also some chemical observations of a qualitative nature made by Schulze in 1856 and by Schlossberger in 1856. Vernon (1895), who made extensive observations on the phenomena of respiratory exchange in various marine invertebrates, thinks that "the solid organic constituents may not consist by any means of pure proteid."

From the above we see that the true nature of the jelly or ground substance of the medusæ is not determined; this makes it difficult to interpret the various changes noted in the present experiments. It is at once evident, however, that the solids can not be constituted as a whole by the ordinary protein, since the latter usually yields nitrogen to the extent of 12 to 18 per cent, contrasted with 3 per cent in the dried *Cassiopea*.

Even if we calculate the nitrogen in ash-free solids, the value rises no higher than 6 per cent; but this nitrogen value (about 6 per cent) is interesting in view of the fact that it is the value found by Schlossberger (1856) for the chitinous substance. Although the nitrogen-content in the solids alone is not adequate to show that we are dealing with a chitin-like substance, yet recent investigations by Henze (1908) on the identity of the framework of siphonophoræ with chitin and, further, the wide occurrence of chitin in numerous marine invertebrates as shown by Irwin (see Armstrong, 1912) indicate that the ground-substance of the medusæ might be similar. If this supposition turns out to be true, its biological significance becomes at once evident, since chitin is closely related to mucin, which in turn is closely related to the cartilage of the vertebrate skeleton.

EXPERIMENTS ON STARVATION.

LOSS OF WEIGHT IN STARVING CASSIOPEA.

Eight freshly caught normal cassiopeas having different body-weights were subjected to starvation by placing them in filtered sea-water. The filtration was effected with all the precautions adopted by Mayer (1914) and the water (4,500 c.c.) in the vessel was changed once every day. The period of starvation was 21 days and the body-weights were taken every day for the first 6 days and then at 10 and 21 days. The loss of weight is shown in table 4.

TABLE 4.—*Loss of weight in starving Cassiopeas.*

Serial No.	Body-weight of specimen.								
	June 28, 0 day.	June 29. 1 day.	June 30, 2 days.	July 1 3 days.	July 2, 4 days.	July 3, 5 days.	July 4, 6 days.	July 8, 10 days.	July 19, 21 days.
	grams.	*grams.*	*grams.*	*grams.*	*grams.*	*grams.*	*grams.*	*grams.*	*grams.*
3	164.15	141.48	131.20	123.32	117.19	113.80	104.48	99.34	62.50
2	156.01	133.42	123.25	115.91	109.43	102.41	95.10	90.40	53.39
1	152.09	132.05	120.42	110.64	100.29	95.00	88.24	83.32	47.80
Average	157.42	135.65	124.95	116.62	108.97	103.74	95.94	91.02	54.56
Per cent	100.00	86.17	79.38	74.08	69.22	65.90	60.95	57.82	34.66
4	97.37	85.53	79.38	71.59	68.80	64.27	57.45	48.74	26.35
5	71.50	64.23	61.78	58.76	54.22	49.97	44.20	36.20	17.51
7	58.89	51.42	49.57	47.01	44.53	40.85	36.09	29.17	16.53
Average	75.92	67.06	63.57	59.12	55.85	51.70	45.91	38.04	20.13
Per cent	100.00	88.33	83.74	77.87	73.57	68.10	60.47	50.10	26.52
6	41.08	35.54	33.46	30.72	28.99	27.01	24.58	18.73	9.22
8	29.46	24.99	23.75	21.94	18.47	15.98	15.41	13.01	5.83
Average	35.27	30.26	28.61	26.33	23.73	21.49	20.00	15.86	7.52
Per cent	100.00	86.08	81.12	74.65	67.28	60.93	56.71	44.97	21.33
Total average	96.32	83.58	77.85	72.49	67.74	63.66	58.19	52.36	29.97
Per cent	100.00	86.77	80.83	75.26	70.34	66.19	60.42	54.36	31.11

These eight cassiopeas were divided into three groups composed of the three largest, the three medium, and the two smallest individuals respectively. The object of such grouping was to see whether or not the loss differed according to the size of the animal. Table 4 shows a distinct difference in the loss according to the initial body-weight. In general, the smaller cassiopeas lost relatively much more than did the larger (see table 5). This result agrees with the results found for the starving vertebrates. It is remarkable, however, that cassiopeas after losing nearly 62 to 80 per cent of their initial body-weight appear in no respect different from the normal cassiopea of similar body-weight. It is very difficult to distinguish the starved from the normal by merely looking at them unless the velar lobes happen to be turned upward—as occurs in some of the cassiopeas after several days of starvation.

Mayer (1914) found a simple law which governs the loss of weight in starving cassiopea. The law is represented by the following formula

$$Y = W(1-a)^x$$

where Y represents body-weight W after x days of starvation, and a stands for the constant that gives a fair measure of the rate of loss of weight. Mayer calls this constant a the coefficient of negative metabolism, since a increases as the loss of weight increases.

Extensive data given by Mayer show that this formula accords satisfactorily with his observed data. The starved cassiopeas in my hands showed, however, a sharp drop in the body-weight within 24 hours after commencing starvation; thus the succeeding body-weights were altogether too low as compared with the calculated values, when this formula was applied. Assuming that the initial sudden loss in weight was due to some peculiar conditions not understood at present (possibly the escape of algæ, etc.), I have taken the body-weight, 83.58 grams (see table 4), one day after starvation, as the starting-point, and computed the successive losses of weight according to the formula[1]

$$Y = 83.58\ (1-0.05)^{x-1}$$

Table 5 shows the observed losses of body-weights (from table 4) compared with those calculated by the revised formula.

Table 5.

Days.	Observed body-weight.	Calculated body-weight.	Days.	Observed body-weight.	Calculated body-weight.
	grams.	*grams.*		*grams.*	*grams.*
1	83.58	83.58	5	63.66	68.08
2	77.85	79.40	6	58.19	64.68
3	72.49	75.43	10	52.36	52.68
4	67.74	71.66	21	29.97	29.97

By this modification the agreement between the observed and calculated values becomes much more satisfactory. The somewhat lower body-weight obtained in the present experiment as compared with that calculated was probably due to the frequent handling of the animal in order to determine the body-weight daily. The removal of the adherent moisture with filter paper previous to weighing is certainly severe treatment for the delicate medusan body. It is therefore rather a surprise to find that the formula, which has no additional provision for the various experimental conditions except the two factors (number of days and initial body-weights), can represent so closely the data here obtained.

[1]The discrepancy between my formula and that of Mayer is due to the fact that Mayer did not commence his observations until after the cassiopeas had remained in aquaria in the laboratory for one day (24 hours), and thus until after all undigested food had been discharged from the medusa. The considerable loss during the first day is largely due to this fact, and also to the loss of much slime soon after being captured.

ANALYSIS OF STARVED CASSIOPEA.

Soon after the final body-weights were determined the specimens were dried in an oven for three days in order to drive out most of the moisture, and then were brought back to the Wistar Institute, where the final drying process was completed two weeks later at 95° to 97° C. The analysis was then made and the results are shown in table 6.

TABLE 6.—*Loss in body-weight and relations of water, solids, and nitrogen in the starved Cassiopeas.*

Serial No.	Initial body-weight.	Final body-weight.	Loss.	Weight of dry solids.	Water.	Solids.	Total nitrogen.	Nitrogen in solids.
	grams.	*grams.*	*p. ct.*		*p. ct.*	*p. ct.*	*mgm.*	*p. ct.*
8	29.455	5.829	80.21	0.305	94.77	5.23	11.06	3.626
6	41.081	9.217	77.56	.467	94.93	5.07	13.58	2.903
7	58.885	16.534	71.92	.847	94.88	5.12	28.56	3.372
5	71.500	17.507	75.52	.970	94.46	5.54	.....	.....
4	97.372	26.351	72.94	1.352	94.87	5.13	39.62	2.930
1	152.092	47.798	68.57	2.391	94.99	5.01	61.60	2.576
2	156.010	53.991	65.39	2.630	95.13	4.87	61.88	2.353
3	164.145	62.495	61.92	3.107	95.03	4.97	82.46	2.654
	[1]96.317	[1]29.965	[1]71.75	.....	[1]94.88	....	.....	.....

[1]Averaged values.

PERCENTAGE OF WATER.

The percentage of water is practically identical in the starved cassiopeas of all sizes, and the average value is 94.88 per cent, which is slightly higher (0.73 per cent) than the average values obtained from the normal cassiopea. A part of this difference may be explained by the fact of the more complete drying of the starved medusa under the favorable conditions of the Institute laboratory as compared with the difficult conditions at the Tortugas. However, as the data given by Mayer also show a slight difference in the water-content between the normal (95.1 per cent) and starved (95.3 per cent), it seems reasonable to conclude that starvation tends to give a slightly higher percentage of water.

PERCENTAGE OF NITROGEN IN THE SOLIDS.

As in the case of the normal cassiopea, the nitrogen-content is highest in the smaller animals and tends to decrease with increasing body-weight. The value of the nitrogen is, however, much higher in the starved cassiopea than in the normal cassiopea of similar body-weight (see table 7).

Thus, 5.56 grams of the normal series gives 2.98 per cent of nitrogen contrasted with 3.63 per cent of nitrogen in the starved cassiopea having a body-weight of 5.83 grams. Such a relation is clearly shown in the majority of cases. It is highly interesting to observe that the percentage

of nitrogen is notably high for the final starved body-weight when compared with that in the control with a similar body-weight (table 7), but it is much lower for the initial unstarved body-weight, as will be seen from the extremely low percentage of nitrogen when calculation was made in respect to the initial body-weight instead of the final (see table 7, sixth column). This indicates clearly that during starvation the animal has utilized a considerable amount of the nitrogenous substances—a conclusion which is important, as it answers the main question before us and brings *Cassiopea* in line with the vertebrates in its reaction to starvation conditions. It is highly probable that some fraction of the nitrogen utilized may represent that of the cellular substance. Mayer (1914) noted,

TABLE 7.—*Percentage of nitrogen in the normal Cassiopea compared with that in the starved Cassiopea.*

Normal.		Starved.				Remarks.
Body-weight.	Nitrogen in solids.	Nitrogen in solids.	Body-weight, final.	Body-weight, initial.	Nitrogen in solids.	In calculating the percentage of nitrogen in columns (2), (3), (6), the solids were estimated by assuming that the 94.16 per cent of fresh body-weight is represented by the water. The total nitrogen in milligrams found in the solids of the starved cassiopeas was compared with the solids thus obtained at the beginning of the starvation experiment and the percentage values were then computed.
grams.	*p. ct.*	*p. ct.*	*grams.*	*grams.*	*p. ct.*	
5.56	2.98	3.63	5.83	29.46	0.64	
10.06	3.00	2.90	9.22	41.08	.56	
[1]15.73	2.53	3.37	16.53	58.89	.82	
[2]109.35	2.36	2.65	62.50	164.15	.86	
(1)	(2)	(3)	(4)	(5)	(6)	

[1]Average of two cassiopeas. [2]Data given by Mayer (1914).

from the sections made on the starved cassiopea, not only reduced cell size, but also many degenerated cell-bodies, and there was evidence also of a complete disappearance of some of the cellular elements. Mayer found also a vacuolated condition of the gelatinous substance accompanied by a greater loss of the muscular tonus. The chemical alterations noted above accord with his findings.

Mayer concluded that the percentage of nitrogen in the solids is independent of the period of starvation and is practically identical with that obtained from the non-starved cassiopea. I have, however, found, as stated above, that starvation tends to increase not only the percentage of nitrogen in the solids, but also that the absolute amount of nitrogen shows an increase when the starved cassiopeas were compared with the normals having the same body-weight. The discrepancy between the conclusions drawn by Mayer and by myself is, I believe, due to the fact that Mayer's observations were limited to the larger cassiopeas (body-weights over 100 grams) in which the percentage of nitrogen in the solids shows little variation following the large variations of the body-weight, while the variations in the nitrogen are quite noticeable in the cassiopeas of smaller size. I may add here that

the data given by Mayer[1] show also a slight indication of a difference in the nitrogen-content between the normal and starved cassiopeas; thus the nitrogen-content in the normal is 2.35 per cent for a body-

TABLE 8.—*Data on the content of water, solids, and nitrogen in three parts of starved Cassiopeas.*

Serial No.	Parts.	Weight, fresh.	Weight, dry.	Water.	Solids.	Total nitrogen.	Nitrogen in solids.
		grams.	*grams.*	*p. ct.*	*p. ct.*	*mgm.*	*p. ct.*
8	Mouth-organs.....	2.847	0.154	94.59	5.41	5.60	3.636
	Umbrella.....	2.320	.118	94.91	5.09	3.64	3.085
	Velar lobes.....	.662	.033	95.16	4.84	1.82	5.515
	Total.....	5.829	.305	94.77	5.23	11.06	3.626
6	Mouth-organs.....	5.249	.269	94.88	5.12	7.98	2.966
	Umbrella.....	3.296	.160	95.15	4.85	3.78	2.300
	Velar lobes.....	.672	.038	94.35	5.65	1.82	4.789
	Total.....	9.217	.467	94.93	5.07	13.58	2.903
7	Mouth-organs.....	9.552	.509	94.67	5.33	18.48	3.631
	Umbrella.....	5.561	.262	95.29	4.71	6.72	2.565
	Velar lobes.....	1.421	.076	94.65	5.35	3.36	4.421
	Total.....	16.534	.847	94.88	5.12	28.56	3.372
4	Mouth-organs.....	16.122	.825	94.88	5.12	23.66	2.868
	Umbrella.....	8.193	.412	94.97	5.03	10.92	2.650
	Velar lobes.....	2.036	.115	94.35	5.65	5.04	4.382
	Total.....	26.351	1.352	94.87	5.13	39.62	2.930
1	Mouth-organs.....	26.669	1.335	94.99	5.01	35.42	2.653
	Umbrella.....	19.420	.954	95.09	4.91	21.00	2.201
	Velar lobes.....	1.709	.102	94.03	5.97	5.18	5.078
	Total.....	47.798	2.391	94.99	5.01	61.60	2.576
2	Mouth-organs.....	31.398	1.529	95.13	4.87	36.82	2.408
	Umbrella.....	18.683	.891	95.23	4.77	17.92	2.011
	Velar lobes.....	3.910	.210	94.63	5.37	7.14	3.400
	Total.....	53.991	2.630	95.13	4.87	61.88	2.353
3	Mouth-organs.....	36.214	1.813	94.99	5.01	49.42	2.725
	Umbrella.....	21.796	1.041	95.22	4.78	22.82	2.192
	Velar lobes.....	4.485	.253	94.36	5.64	10.22	4.040
	Total.....	62.495	3.107	95.03	4.97	82.46	2.654
5	Mouth-organs.....	9.212	.506	94.51	5.49	.....	.....
	Umbrella.....	7.297	.367	94.98	5.02	.....	.....
	Velar lobes.....	.998	.061	93.89	6.11	.....	.....
	Total.....	17.507	.934	94.46	5.54	.....	.....

weight of 109.3 grams, while the starved gives 2.47 per cent of nitrogen for a final body-weight of 92.4 grams. These differences agree with my own findings.

[1]Mayer, A. G. 1914. The law governing the loss of weight in starving *Cassiopea*. Carnegie Inst. Wash. Pub. No. 183, table 2, p. 59.

I am thus inclined to believe that even these larger cassiopeas may show a significant degree of alteration after a longer period of starvation than that given them by Mayer, and when the animals have lost 80 per cent or so of their initial body-weight.

ANALYSIS OF THE DIVISIONS OF THE STARVED CASSIOPEA.

Various determinations made on the different parts of the body show that the water-content is practically identical in all the starved cassiopeas. Here also, as in the case of the normal animal, the umbrella gives a slightly higher water value, owing possibly to the inclusion of the stomach contents (water). The percentage of nitrogen in the solids also shows relations similar to those in the normal cassiopea; that is, the nitrogen-content is highest in the velar lobes and lowest in the umbrella, while that for the mouth-organs occupies an intermediate position.

I have shown (in table 6) the loss of weight in body as the result of 21 days of starvation. We noted that in this series the cassiopeas lost on the average as much as 72 per cent of their initial weight, but as to whether they lost in weight proportionally in the different divisions of the body, or whether the loss was otherwise distributed in the three parts, I have found the following relations:

For a final starved body-weight of 29.97 grams (table 6) the relative weights of the mouth-organs, umbrella, and velum are 18, 10, and 2 grams, respectively, when read from figure 1, while the corresponding observed weights were 17.16, 10.82, and 1.99 grams. Reducing these relative weights to ratios we obtain for the body-weight of 29.97 grams

From figure 1, 9:5:1 From observations, 8.6:5.4:1

For the initial body-weight of 96.32 grams (table 6) of the animals subsequently starved to the weight just noted the ratios (from fig. 1) are 8:5:1. Considering the conditions under which the comparison of these data are made, these three ratios may be regarded as equivalent and show that the three parts of the body have, as it were, reversed the process of growth during starvation. The strictly proportional loss in these three parts accounts for the normal appearance of the animals, even after they have lost as much as 62 to 80 per cent of their original weight.

The statement made in my preliminary report (1916) that "in the starved cassiopeæ the relative weights of the three parts coincide with those given by the normal cassiopea, which have a body-weight similar to the starved cassiopea at the end of the test, and not to the relative weights at the beginning of the experiment," should therefore be changed to a more general statement by omitting "and not to the relative weights at the beginning of the experiment," since the deviations of the proportional values after starvation are so slight, whether

these are compared with the proportions given by the normal examples having either the final or initial body-weights. More exact statements can be made after we have obtained a large number of data on both the normal and starved specimens.

Briefly summarized, the results of the observations made on the starved cassiopea are as follows:

(1) In general the smaller cassiopea loses relatively more in weight than does the larger cassiopea.
(2) The percentage of water found in the entire body, as well as in the three different parts, is nearly the same in all sizes of cassiopea. However, the values of the water-content in the starved appear to be slightly higher than that found in the normal cassiopea.
(3) The nitrogen-content of the entire body is higher in the small than in the larger cassiopea, as in the case of the normal animals.
(4) However, the absolute amount of nitrogen found in the starved cassiopea is considerably higher than in the normal having the same body-weight. It was noted also that, although high when compared with a normal specimen equal in weight to the starved animal, it is very low for the initial body-weight of the starved animal. This shows that the nitrogen has also been consumed during the period of starvation.
(5) The nitrogen-contents for the different parts of the body are similar in their relations to those found in the normal cassiopea.
(6) The loss in weight of the different parts is of such a character that their proportion in the starved remain similar to those in the normal cassiopea.

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VI.

THE HABITS AND REACTIONS OF A COMATULID, TROPIOMETRA CARINATA.

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THE HABITS AND REACTIONS OF A COMATULID, TROPIOMETRA CARINATA.

By Hubert Lyman Clark.

INTRODUCTION.

One of the most interesting echinoderms taken during the recent visit of the Carnegie Institution's party to Tobago (March and April 1916) was the comatulid *Tropiometra carinata* (Lamarck). This crinoid was common in Buccoo Bay, and while Dr. Mortensen successfully undertook the study of its development, I made such observations on its habits and reactions as time and circumstances permitted. The habitat of most of the individuals noted was so completely different from any in which we had previously seen comatulids that we naturally inferred the reactions would differ markedly from those of the species studied in Torres Strait.[1] I therefore performed a few simple experiments, the results of which are here recorded, together with such observations as were made on the animal in its natural environment.

SYSTEMATIC POSITION.

Comparison of the comatulids taken at Tobago with a considerable series of specimens from several stations on the Brazilian coast, south of the Amazon, shows that they are unquestionably identical. For this Brazilian species, Mr. A. H. Clark has revived an old name of Gay's, *picta*, regarding it as "a perfectly good species," "most obviously differing from *carinata* in the greater length of the outer cirrus segments" (Proc. U. S. Nat. Mus. 1911, vol. 40, pp. 35, 36). On comparing the Brazilian and Tobagoan material with specimens from Mauritius and Zanzibar, I was utterly unable to detect any differences, either in the cirri or in any other characters. On writing to Mr. Clark of my difficulties, he very kindly replied that while the species of *Tropiometra* are very difficult to distinguish, he finds "no difficulty in distinguishing" the group in which he places *picta* from that in which he places *carinata*, "by the difference in length of the outer cirrus segments." On receipt of his letter, I went over the cirri again, but I found myself absolutely unable to detect the difference named. I have therefore very reluctantly reached the conclusion that I can not recognize *picta* as a valid species, but must designate my Tobagoan comatulids by their old Lamarckian name.

[1]Comatulids of Torres Strait, H. L. Clark, Carnegie Inst. Wash. Pub. No. 212, pp. 95–125, 1915.

HABITAT.

We first found *Tropiometra* in Buccoo Bay, where it occurs in water from a few inches to several feet in depth at low tide; indeed, at the lowest tides some individuals are probably out of the water, in part at least, for a short time. The bottom which they frequent is made up largely of *Porites* fragments, usually more or less covered by a growth of *Corallina* and *Halimeda*. Scattered over it there is also a sparse growth of short eelgrass (*Zostera*). As a rule the comatulids hold themselves in an erect position by means of their stout cirri, which are customarily grasping a bit of *Porites*. Sometimes the body is more or less completely shaded by a clump of eelgrass or seaweed, but this is not usually the case. Generally the individuals are solitary, but occasionally five or six may be found about a single clump of *Porites* or of eelgrass. They are not really abundant, but twenty or more may be gathered in half an hour or less. Now and then we found individuals living underneath slabs and large fragments of coral; this was particularly true on Buccoo Reef, where, later, a number of specimens were discovered. All were in shaded places, suspended arms down, and not in the usual erect position.

COLOR AND SIZE.

The first specimens of *Tropiometra* seen appeared to be uniformly dark purple, purplish-brown, reddish-brown, or bright brown, but a closer examination showed that uniform coloring was very rare. Almost always a longitudinal dorsal stripe on each arm, or transverse bands of more or less width, or both, are present. The color of these markings is yellow of some shade, often dull and buffy, but not rarely quite bright. In some individuals the pinnules are also cross-banded with yellow, and the distal pinnules may be uniformly brownish yellow. The cirri are yellow brown, at least dorsally, but are often more or less dusky or purplish on the oral surface; in nearly all individuals, however, both young and old, the terminal two or three segments (except the claw) have a dusky spot on the oral side. This marking seems to be a very constant character in Tobagoan specimens. Occasional individuals are found in which the pinnules and dorsal side of the arms are plenteously besprinkled with silvery-white, giving them an exceptionally handsome appearance.

All of the small specimens found were brownish-yellow or bright brown, more or less marked and banded with purple, and this general coloration is not rare in adults, particularly in those found under slabs of rock on Buccoo Reef and in similar shaded places. Some of these individuals were very handsome in their brilliant array of purple and gold, and it was hard to believe they were really identical with the dull-colored specimens from the shallows of Buccoo Bay. A natural

inference from the specimens seen is that the young are uniformly yellow[1] or brownish-yellow, and that the purple pigment develops as they mature, in some individuals completely obliterating the original color, but usually appearing simply as spots, blotches, and cross-bands. One could scarcely avoid the impression that the development of the pigment is associated with life in the open sunlight, but there was no chance to secure an answer to the interesting question which suggests itself: Do the bright-colored individuals avoid the sun because they lack pigment or do they lack pigment because they have never lived exposed to the sun?

Several very small individuals were found under rocks on Buccoo Reef, but the smallest one seen (having arms about 18 mm. long) was discovered in a clump of *Corallina*. The largest specimen measured had an arm-length of nearly 100 mm., and a few specimens exceeded that, but the great majority had arms 60 to 80 mm. long. No specimens with more or fewer than ten arms were noted, in more than 200 examined, but several cases of arms forked distally were seen and in one case a forked pinnule was noted. As a rule the arms were approximately equal, but in some individuals those of one side were distinctly shorter than the others. In such cases, however, it was usually obvious that the short arms were regenerating.

FOOD.

Although some individuals live suspended under rocks with the mouth down, while the great majority are erect with the opening upwards, the character of the food is in all cases planktonic—that is, it does not consist of such organic matter as happens to fall on the disk and arms, but is made up of the living, active plankton. When examined under the microscope, the stomach contents are seen to consist of a mixture of vegetable and animal food, the former predominating.

The plants are diatoms and unicellular green algæ, with occasional fragments of other seaweeds. The condition of the green algæ showed that most, if not all, were ingested while living, and the same seemed to be true of the diatoms. Of animals, crustacea were most frequently noted, but a few foraminifera were also seen. The crustaceans were minute amphipods, copepods, and crab zoæas, and all were in such condition as to leave no doubt that they were alive when ingested. Just how such active animals are actually captured and forced into the mouth is not clear. As compared with the comatulids studied in Torres Strait, *Tropiometra* shows an unusual proportion of animal food in its diet, but this may of course be purely a local or seasonal matter.

[1]In support of A. H. Clark's views on the color of young crinoids. See Geog. Jour. London, Dec. 1908, p. 606.

LOCOMOTION.

Like most of the comatulids hitherto studied, *Tropiometra* is exceedingly sluggish. So far as my observations go, it never swims. Dropped or suspended in deep water, it makes only the most feeble arm-movements and, unless artificially prevented, it sinks rapidly to the bottom. When, as a result of some adequate stimulus, *Tropiometra* does move, the movement is similar to that of *Comanthus*. That is, it is accomplished solely by means of the arms, and no particular arms take precedence in the matter. In one respect the attitude differs from that of *Comanthus*, for the body is much more distinctly raised. This is obviously to keep the cirri wholly above the substratum and thus prevent their accidentally catching on it. The movement is always slow and labored and does not continue long. When overturned so that the cirri are up, a normal healthy *Tropiometra* invariably rights itself at once, sometimes only very slowly and deliberately, but usually without delay. The process is, as in other comatulids, by several arms raising up their side of the body until one or more of them or some of the neighboring arms can reach over backwards and secure a hold which will enable them to pull the body over. The cirri play no part whatever in this process, any more than in actual locomotion; they are very inert, move slowly and slightly, and are used only as organs of attachment, a purpose which they serve admirably. The pinnules, even near the tip of the arm, have only weakly hooked tips and are but slightly viscid, and it is consequently difficult for *Tropiometra* to move or even to right itself except on a rough and rather firm surface. No individuals were seen moving about under normal conditions, but when placed on a bare, sandy bottom they crept steadily to its margin of coralline algæ. Many specimens were planted on the reef-flat near the laboratory and most of these ultimately disappeared, but whether they crept away or were carried off by tidal movements it is impossible to say.

REACTIONS TO LIGHT.

The remarkable habitat of *Tropiometra* in Buccoo Bay naturally suggested that in its reactions to light it would be positively phototactic. A tank 6 feet long, a foot wide, and 8 inches deep, having the bottom covered with broken coral and stones, was used for the experiments. It was placed near high-water mark on the beach, in the sun, and two boards, 5 or 6 inches wide, were laid across it, giving shaded areas; one end was made quite dark by covering with a broad piece of board and burying it in the sand. Numerous individuals of *Tropiometra* were tested in this tank on many different occasions and under diverse conditions of temperature. The results were surprisingly unsatisfactory. No individual showed any decided reaction to light and none sought the darkness. In most cases, when the specimen

was fresh from Buccoo Bay, if placed in a shaded area it remained there; if placed in the sun close to a shaded area, it moved into the shade, but if the board were then removed, it made no effort to move out of the sun; if placed in the sun some inches from shade, it remained quietly in the light. Generally the individuals were placed upside down in the tank, so that the process of righting might serve to initiate movement. But although they righted themselves promptly, they rarely wandered more than a few inches thereafter. If placed in the tank right side up, they were usually content to remain where put, regardless of whether it was in sun or shade.

Experiments were made particularly to see whether the brightly colored specimens from under rocks reacted differently from the dark individuals from Buccoo Bay, but no constant differences could be detected. Brightly colored examples of *Tropiometra* seemed to be a little less hardy and became sluggish and inert in the tank more quickly than the dark ones, but the difference was not striking. The final conclusion reached, as a result of all the experiments and observations, is that *Tropiometra* at Tobago is *slightly negatively phototactic*, but that other factors in the environment in Buccoo Bay neutralize this tendency almost or quite completely, while on Buccoo Reef it is for some reason strengthened.

REACTIONS TO TEMPERATURE.

While it was not feasible to produce really low temperatures, water in the tank fell to 22° C. during the night. At this temperature *Tropiometra* showed no signs of discomfort or stimulation. When the water was gradually raised to 33° or 34°, responses to mechanical stimulation were normal. At 35.5° two of the four specimens tested were quite unresponsive, but the other two gave normal responses by arm-movements. One continued to respond at 36.5°, but at 37.5° was inert. After a few moments at that temperature the four specimens were placed in normal sea-water at 28°, but none recovered. Individuals taken from water at 28° and placed in that at 33° continued to give responses with the arms. At 34°, however, the arms were quiescent, but the pinnules responded. Complete recovery of these specimens resulted when after a few moments they were again placed in water at 28°. Individuals placed in water at 36° gave no responses and failed to recover; they became quite rigid. These experiments show that as compared with the comatulids of Torres Strait, *Tropiometra* has a surprisingly large temperature range. This is quite in keeping with their habitat in Buccoo Bay, for while the water there probably never falls below 22° C., there is no doubt that at the lowest tides it rises in the shallows to 32° and possibly to 34°. Even should it on rare occasions exceed this figure for a short time, it is evident that *Tropiometra* would easily survive. Owing to the comat-

ulid's inertness it was impossible to determine the optimum temperature. In water of 33° reactions were not consistently different from what they were at 25°; nevertheless individuals in water at 33° assumed a somewhat wilted appearance after a time, which was not the case in water at 28° and lower. Healthy individuals gave no response to a sudden change of 2°, but there was generally an evident reaction to an abrupt change of 5°.

REACTIONS TO SALINITY.

Experiments to determine whether *Tropiometra* is sensitive to changes in the salinity of the water in which it lives gave very interesting results. Two individuals were placed in an aquarium containing 5 liters of ordinary sea-water and two specimens of similar size and appearance were placed in another aquarium containing 5 liters of 90 per cent sea-water (*i. e.*, 4.5 liters of sea-water plus 500 c.c. of rain-water). After 12 hours all were normal and one could not tell from the responses to mechanical stimuli which were in the diluted sea-water. Similar experiments were tried with water only 80 per cent and 75 per cent sea-water. Such water extracted color from the comatulids and after 12 hours was distinctly yellow, yet the tropiometras survived and responded to the transfer to normal sea-water by distinct arm-movements.

Experiments were then made to see how concentrated a sea-water could be survived. Two specimens, one dark-colored and one yellow and purple, survived with no apparent injury 12 hours in only 2 liters of water 10 per cent more saline than normal. The brightly colored one was more active after the experiment than the other, which was not what I had expected in view of the greater evaporation in the natural habitat of the dark specimen. A similar experiment with water 20 per cent more saline than normal was tried. A brightly colored individual was still alive after 3 hours; it was then transferred to normal sea-water and lived over night. A dark-colored specimen was still alive after $4\frac{1}{2}$ hours, but although it was then transferred to normal sea-water, it failed to revive. These experiments show a surprising indifference on the part of *Tropiometra* to the salinity of the water in which it lives.

REACTIONS TO CERTAIN CHEMICALS.

Although no experiments were made to test the reactions to chemicals, it may be worth while to record the observations made in other connections. Indifference to the amount of CO_2 in the water was noticeable, particularly in view of the sensitiveness of Torres Strait comatulids to that substance. As already stated, 2 specimens lived over night in only 2 liters of sea-water, apparently without discomfort, and 4 or 5 specimens were often kept over night in aquaria with not

more than 6 or 7 liters. Unlike the comatulids tried in Torres Strait, *Tropiometra* when fresh from the sea responds to a saturated solution of $MgSO_4$ in sea-water, like other echinoderms, the whole muscular system becoming relaxed. The response was much less complete if the individual had been for some time in a limited amount of sea-water; probably the presence of CO_2 made the difference. Response to alcohol and formalin was, as in other comatulids, by a contraction first of the dorsal side of the arms and then of the ventral. Only exceptionally, and then by individuals which had been long in tank or aquaria, were the arms cast off.

SUMMARY.

The common habitat of *Tropiometra* at Tobago, Buccoo Bay, is unusual for a crinoid, the bottom being covered with coralline algæ and eelgrass, the water shallow, and the comatulids exposed to the full glare of the tropical sun.

Reactions to light, temperature, salinity, and CO_2 all show *Tropiometra* to be remarkably insensitive and hardy. It shows only feeble phototactic reactions, has a temperature range of at least 15° C., a salinity range well over 40 per cent, and withstands a considerable but undetermined amount of CO_2 without injury.

VII.

STUDIES ON THE PHYSIOLOGY

OF THE

NERVOUS SYSTEM OF CASSIOPEA XAMACHANA.

By LEWIS R. CARY,

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Eighteen text-figures.

STUDIES ON THE PHYSIOLOGY OF THE NERVOUS SYSTEM OF CASSIOPEA XAMACHANA.

By Lewis R. Cary.

INTRODUCTION.

In this paper are gathered the results of several distinct lines of experimentation, all of which have this in common—that they deal with some phase of the physiology of the nervous system of *Cassiopea* and represent portions of a general program of research on the nervous system of the lower animals. On account of its ability to live under adverse conditions and to withstand practically any type of operation, *Cassiopea* is an especially favorable form for experimentation and has been used as a subject for many researches. On regeneration by Stockard, Zeleny, and Goldfarb; for the study of pulsation, rate of nerve-conduction, effect of starvation, etc., by Mayer; on chemical composition and chemical changes during starvation by Hatai; on reaction to temperature, permeability, and nerve-conduction by Harvey, and the researches herein recorded by the author.

In very few types of animals, where the recognizable activities are entirely under the control of nerve-centers, can all these structures be removed without causing death, or at least serious injuries which will render abnormal practically all physiological activities. Among the Scyphomedusæ, *Cassiopea*, in common with many other Rhizostomæ, is distinguished for the density of its mesoglœa, but it stands preeminent as a laboratory marine animal because of the fact that its normal habitat, being in shallow lagoons of relatively stagnant water, fits it especially for the adverse environment encountered under experimental conditions in laboratory aquaria. The sense-organs (nerve-centers), because of their position around the periphery of the disk, can be removed with very little injury to any of the other tissues of the body.

Since, also, these structures are equally spaced around the circumference of the disk, any portion of the body can, by the appropriate operation, be deprived of its normal nerve-supply, while retaining its other relations undisturbed. Then, too, the rapidity with which the centers are replaced by the regeneration of normally functioning new ones permits experimenting on any given area of tissues, first without and later under the influence of nervous impulses.

In studying regeneration in *Cassiopea* I found that the rhopalia exert a decided influence on the rate of regeneration, which is most marked in the early stages of any experiments, and that this influence was in the major part independent of muscular activity. The observations were, therefore, extended to involve other factors as the basis of comparison, since it seemed evident that the influence on regeneration must be in some manner exerted through fundamental metabolic activity, which might be expected to be measurable on some other basis than the rate of regeneration. These expectations were fulfilled when either the general metabolism, as measured by total CO_2 production, the loss of weight during starvation, or the changes in the rate of nerve-conduction in response to changes in temperature, was used as the standard of comparison.

The same types of operations were used in experiments on regeneration, general metabolism, loss of weight during starvation, and the influence of the sense-organs on the change in rate of nerve-conduction in response to changes in temperature, so that the results obtained by use of these distinct standards of measurement are directly comparable.

The experiments on the influence of the nerve-centers on the loss of weight during starvation, on account of the operations employed, necessarily involved regeneration at the same time, and in some instances this factor was measured as well as that for which the experiments were primarily carried out. Conversely the experiments on regeneration, since only the disks were used, were carried out on starving medusæ and the decrease in both area and weight of the half disks was often recorded for these experiments. While both these factors (*i. e.* regeneration and decrease in size and weight) were involved in the experiments on the rate of nerve-conduction, each experiment extended over so short a period of time that no measurements of the amount of regeneration were possible, and the loss of weight, while actually small, was entirely in accord with the results obtained from the more extended experiments. Thus the high rates of pulsation brought about by nerve-conduction, independent of the rhopalia, tended especially to emphasize the inadequacy of differences in motor activity as the explanation of difference in rate of metabolism as expressed by loss of weight.

TECHNIQUE OF THE EXPERIMENTS AND SOME RESULTS OF OPERATIONS THAT APPLY EQUALLY TO ALL EXPERIMENTS.

The natural habitat of *Cassiopea* is in shallow lagoons, usually in areas where mangroves are abundant, where the water is daily subjected to marked fluctuations in temperature, and where the salinity is most affected by precipitation. In such locations, many of which are practically stagnant, the variations in salinity are of wide range, as evaporation is rapid during the summer months, when also the precipitation is greatest. Such lagoons usually support an abundant algal flora, so that the gaseous content of the water varies considerably and large quantities of organic acids are continually being generated. The environmental conditions consequently vary widely from time to time and are always decidedly different from those in the open tropical ocean. Indeed, so thoroughly inured to this environment is *Cassiopea* that it thrives better under laboratory or lagoon conditions than in pure sea-water.

At Tortugas the proper environment for this form is restricted to the moat, at Fort Jefferson, which is 50 feet wide, extends entirely around the fort, and communicates with the open water outside at only two points through narrow entrances which are entirely cut off at extreme low tide. As the mean rise and fall of tides is in this region only about 1.5 feet, the amount of change in the water of the moat is slight and currents are scarcely detectable at a distance of 200 yards from the entrances. Over the greater portion of the moat, the bottom is densely covered with a mat of filamentous algæ, while its side walls furnish a place of attachment for innumerable specimens of hydroids, bryozoa, tunicates, annelids, mollusks, and several species of corals. Among the algæ the cassiopeas lie on their aboral surfaces, with their branching mouth-arms giving a flower-like appearance and with the bell-margin pulsating slowly. In adult specimens, movement from place to place by their own activity apparently occurs very rarely, if at all. Even young medusæ, not more than 2 cm. in diameter, seldom are raised from the bottom by their own pulsation, and it is doubtful if the larger specimens are capable of moving about by swimming movements of the disk.

The exumbrella surface is depressed in the center and can be used as a sucking disk by which a medusa can attach itself firmly to a vertical surface or resist removal from the bottom. This surface has a simple layer of epithelial cells over the mesoglœa and is not provided with either nerves or muscle-cells.

When resting in its normal position on the bottom, the languid pulsations of the bell-margin create currents sufficient to bring the food material (which consists entirely of minute organisms) onto the mouth-

arms, where it is taken in through the numerous small openings into which the original oral cavity has been subdivided by the branching and anastomosing of the oral arms.

When brought into the laboratory the medusæ may be kept in aquaria through which a small stream of water is run slowly—or, if only 3 or 4 specimens are placed in a 10-liter jar, they will remain in normal condition if the water is changed once every 48 hours. The unusual agitation of the water attendant upon changing that in the jars or the handling of the specimens causes a copious secretion of mucus from glands situated on the oral arms, so that the less often they are disturbed the more nearly normal they will remain.

Specimens kept under either of the conditions just mentioned gave the expected results when used for any of the experiments, but in order that any possible source of error from differences of previous treatment might be eliminated, no specimens were used for routine experiments which had been in aquaria more than 12 hours before the time of the first operation.

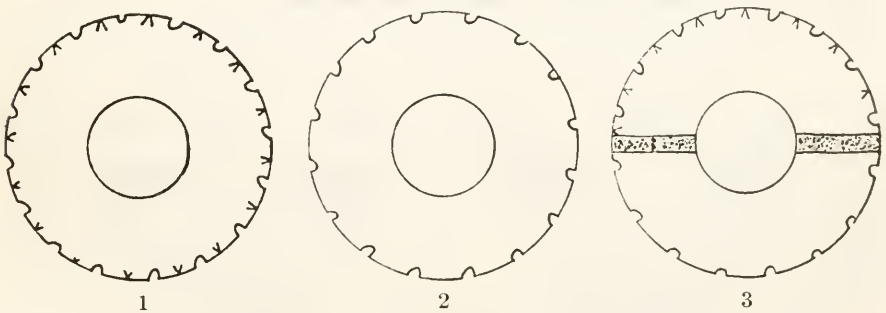
In all experiments which were to extend over more than a few hours, each disk (or its two separated halves) was kept in a jar containing not less than 4 liters of sea-water.

As most of the experiments required the handling of the specimens under observation once each day they were transferred to clean jars of fresh sea-water at that time.

The nervous stimulus causing pulsation originates in the sense-organs, probably through a chemical reaction liberating sodium, at the nerve-centers (Mayer, 1908). At any given time only a single sense-organ, which at that moment is discharging at the highest rate, controls the rate of pulsation. Consequently all the nerve-centers except one may be removed without seriously interfering with the activities of the medusa; when the last one has been removed the specimen will remain quiescent until a new sense-organ has been regenerated with sufficient completeness to take up again the normal chemical reactions which liberate sodium.

Any portion of the disk adjacent to where the nerve-centers have been removed may be insulated from the influence of the remaining centers by destroying the continuity of the subumbrella ectoderm, in which alone the muscles and nervous elements are contained, while still retaining its continuity with the remainder of the disk through the mesoglœa. This mesoglœa is of sufficient thickness to afford a support for isolated areas of the active tissues even when almost the entire ectodermal covering has been removed. Also when the medusæ are kept in normal sea-water it is little subject to bacterial action and can be maintained in an apparently healthy condition even when considerably more than half of the ectodermal layer has been removed.

Three comparisons were made in the experiments dealing with regeneration, loss of weight during starvation, total metabolism, and the influence of the sense-organs on the change in rate of pulsation in response to change in temperature. For all these experiments the oral arms and stomachs were removed and the disk alone used, to avoid the contamination of the water by mucus and to facilitate the operations. In each experiment disks of the same size were used, although specimens of the same diameter showed considerable variation in weight due to differences in the thickness of the mesoglœa. For studying the general metabolism large specimens were used because it was easier to perform the necessary operations. In the first type of experiment, normal disks, or half-disks, were compared with others that had been rendered inactive by the removal of all sense-organs (figs. 1, 2, 3). The sense-organs were cut out from the border of the



Figs. 1 and 2.—Entire medusæ. From fig. 2 all the sense-organs have been removed. From fig. 1 an equal amount of tissue has been removed from between the sense-organs.

Fig. 3.—A medusa prepared with active and inactive halves by removing the sense-organs from half of the disk and then insulating this half from that on which the sense-organs remain by removing two diametrically opposite strips of subumbrella tissue, shown as stippled areas. A piece of tissue was removed from between each pair of sense-organs on the active half-disk. The inner circle represents the limits of the cavity in which the amount of regeneration was measured.

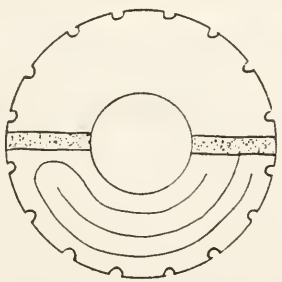
disk by a sharp cork-borer just large enough to completely remove this structure at one stroke. From between the sense-organs of the "active" specimens a piece of tissue of equal size was removed so that the amount of injury was the same for both specimens.

In all the experiments on regeneration recorded in the tables given in this paper the halves of each disk were insulated by the removal of two diametrically opposite strips of subumbrella ectoderm extending from the periphery of the disk on each side to a cavity in the center of the disk where the amount of regeneration was measured (fig. 3). In later regeneration experiments and all other experiments where this type of operation was used the medusa disk was separated into halves and the regular operation was performed upon each half. For measuring the amount of regeneration a disk of tissue 22 mm. in diameter was removed from a similar part of each half-disk. This complete separa-

tion of the half-disks did away with the folding of the disks at the point where the insulating strips were removed, which often interfered with accurate measurement of the regenerated tissue. (These series will be referred to as the "active" and "inactive" series.)

In the second type of experiments normal disks or half-disks were compared with others from which all sense-organs had been removed, but in which the subumbrella muscles had been activated by electrical stimulation. In entire half-disks, from which the central portion has been removed, a circuit wave of contraction may be initiated by induction shocks and maintained for as many as 11 days unless interrupted by some strong stimulus (Mayer, *op. cit.*; Harvey, 1911).

When half-disks were employed it was necessary to make two incisions through the muscles and nerves in such a manner that an endless labyrinth of these tissues would be formed (fig. 4), in which the circuit wave of contraction could be maintained.



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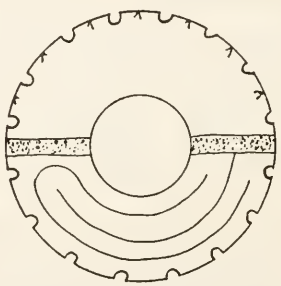

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Fig. 4.—A medusa prepared for comparison of activated and inactive half-disks. All the sense-organs have been removed from both halves, while in the subumbrella tissues of one of them an endless labyrinth has been formed in which a circuit wave of contraction will be maintained as soon as it has been initiated by induction shocks.

Fig. 5.—A medusa prepared for the comparison of active and activated half-disks.

These series will be referred to as the "active and activated" series.

In the third type of experiments "activated" specimens prepared in the manner just described were compared with "inactive" specimens (fig. 5).

In all instances where active and activated specimens were compared it was observed that the pulsation-rate of the activated specimens was at first about 3 times as great as that of the active specimens. The difference in rate became progressively greater for about the first 24 hours, both on account of an actual increase in that of the activated as well as a decrease in the rate of those with sense-organs intact, until at the end of this time the activated specimen was often pulsating 10 times as rapidly as its mate. If an entire disk or unmutilated half disk were used as the active specimen of any pair its pulsations appeared to be much more vigorous than its mate, in the muscles of which a circuit wave of contraction was maintained, because the bell-

margin was folded inward onto the body of the disk at each contraction. When, however, a similar labyrinth had been formed in the subumbrella tissues of the active specimen and the continuity of the sheet of muscles had been destroyed, the bell-margin was no longer folded over and its pulsations lacked the appearance of greater vigor than that shown by the activated specimens.

When simultaneous kymograph records were made of the pulsations of the halves of a disk, one active and the other activated, each with an endless labyrinth formed in its subumbrella tissues, the amplitudes of contraction were found to be equal, showing that the apparent character of the contraction was a function of the normal spatial relationship of the muscle tissue and that when similar series of cuts were made in the active tissues of each half-disk of any pair the rate of pulsation was a true measure of the work done.

Besides a control series carried along as a check for each experiment, a large series of half-disks were prepared, so that each member of any pair (*i. e.*, halves of the same disk) had been subjected to the same operation. Fifty pairs of such half-disks of the three operative types used were recorded for rate of regeneration and loss of weight during starvation, and 10 pairs for total metabolism, with the result that in every instance the similarly prepared halves of each disk gave results which were within the limits of error of the unit of measurements employed in each set of experiments. It is evident from these results that the medusa disks can be safely considered as physiological units which may be subdivided, with the expectation that like areas or masses of tissue of any one disk will respond in an equal degree to experimental changes in environmental conditions.

EXPERIMENTAL.

INFLUENCE OF NERVE-CENTERS ON RATE OF REGENERATION.

Previous studies on the influence of the nervous system upon regeneration have given very divergent results which can hardly be reconciled even when we consider that widely separated groups of animals were used as the material for experimentation.

While certain students of this problem (Herbst, Goldstein, Walter, Wolff) have taken the position that the nervous system in general, or some portion of it (sensory ganglion, Herbst, Walter), exerts a stimulus necessary for the complete regeneration of normal structures, on the contrary other workers have attributed less and less importance to these influences. The intermediate position that the influence of the nervous system is indirect, being exerted mainly through the controlling of motor activity, is well expressed by Child (1905*a*) in the statement concerning anterior regeneration in *Leptoplana*, that, "as in posterior regeneration, there is close parallelism between the rapidity, amount, and completeness of anterior and lateral regeneration and the characteristic motor activity of the part concerned."

Goldfarb (1909) concludes from his experiments on newts, earthworms, and planarians that "these experiments * * * should make one cautious about accepting the view of the direct or even indirect influence of a nervous influence on regeneration."

In all these studies the point at issue has been whether or not complete regeneration of typical structures is possible in the absence of any influences exerted through the central nervous system. An affirmative answer to this question is apparently held, at least by certain of these investigators, to settle finally the question of nervous influence without any consideration being given to the comparison of the course of the regenerative process in animals in which the nervous system was removed from the regenerating area and those in which the nervous system had been uninjured in the portion of the animal left to regenerate. In only relatively few animals can the nerve-centers be removed without bringing about the destruction of, or degenerative changes in, other intimately connected portions of the nervous system, so that this type of operation has not been frequently undertaken.

Zeleny (1907) and Stockard (1908) removed the marginal sense-organs from the disk of *Cassiopea xamachana* to determine the influence of these structures on the rate of regeneration. Both report that there was no evidence of any regulatory influence. In Zeleny's experiments the entire margin of the disk with its sense-organs was removed and the rate of regeneration in these individuals compared with others in which the bell-margin and sense-organs were intact. In Stockard's experiments the results obtained from specimens prepared as above described were supplemented by those obtained with individuals from

which one half of the marginal sense-organs was removed, while from the other half an equal amount of tissue was cut from between the sense-organs. The two halves were insulated by the removal of two diametrically opposite strips of subumbrella ectoderm. In both experiments the rate of regeneration was measured inward from the periphery of a cavity in the center of the disk from which a circular piece of tissue had been removed.

In both these researches the experiments were carried out with the view of ascertaining the influence of muscular activity and thus indirectly of the nervous system on the rate of regeneration. In each case it was held that there was no constant difference in the rate of regeneration between the active and inactive individuals.

In the course of my studies, which were taken up primarily to reëxamine the work of Stockard and Zeleny upon this point, I discovered that the marginal sense-organs influence regeneration independently of their control of muscular activity. Such an influence of the sense-organs can be accounted for either on the ground that metabolic activities, not expressed by muscular activity, are under the control of the sense-organs, or that a direct trophic influence is exerted by the sense-organs on the regenerating tissues. A series of determinations with the "biometer" of the rate of CO_2 production by specimens under different experimental conditions, for which I am indebted to Dr. S. Tashiro, shows that the first of the two alternatives just mentioned offers a satisfactory explanation of the observed facts.

The course of normal regeneration as shown when a disk of tissue is removed from the center of a medusa disk is that first the ectodermal epithelium from both the exumbrella and subumbrella surfaces grows inwardly over the exposed surface of the mesoglœa until that extending from the two surfaces has come in contact. Fusion of the two layers takes place at once and then a sheet of new tissue begins to extend across the cavity left by the removal of the disk of tissue. In this sheet of new tissue no mesoglœa layer is at first distinguishable, the two layers of epithelium being in direct contact at their inner surfaces.

Just as in the embryonic development of all Cnidaria, the mesoglœa makes its first appearance as an acellular layer of gelatinous material secreted by the cells of the layers it is to separate. In later stages of regeneration the newly formed mesoglœa fuses insensibly with that of the body of the disk at the periphery of the cavity. When the sheet of regenerated tissue reaches the center of the cavity its edges fuse, but leave for more than 24 hours a recognizable scar at the point where fusion took place. In the specimens used in the following experiments this scar was at first eccentrically placed, but within the first day after it had been formed it came to lie centrally in the sheet of new tissue. From this time the most noticeable change was the increase in the thickness of the regenerated tissue, which within two weeks—in speci-

mens in sea-water—could not be distinguished from the surrounding tissue. Long before this time tissue differentiation had taken place, so that functional nerves, muscles, gland and sensory cells had been formed from the syncitial mass into which the epithelial layers had been converted in the earliest stages of regeneration.

EXPERIMENTS WITH ENTIRE DISKS.

When the rates of regeneration of a series of active and inactive entire disks is compared it is found that in about 75 per cent of all the experiments the regeneration is most rapid in the active specimens. In the remaining disks the amount regenerated at any given time is (in about 10 per cent of the pairs) found to be equal within the limits of accuracy of measurement, while in about 15 per cent of the pairs of disks regeneration was greatest in the inactive specimens.

The results of many different kinds of experiments upon *Cassiopea* show wide variations in the sensitivity and metabolic activity in this animal. It therefore seems evident that the conflicting results obtained from specimens subjected to this type of operation are to be explained as individual variations in physiological activity.

More dependable results may be expected from specimens prepared according to Stockard's method (fig. 3), where individual variations in physiological activity are eliminated.

After this operation the inactive half of each specimen is moved about by the pulsation of the active half, so that there can be little difference in the degree of aeration of any two parts of the disk. In all experiments of this type (entire disks with the halves insulated from each other) where large numbers of specimens were used two difficulties were met in making the measurements. Frequently the disk became folded backward at the point where the subumbrella ectoderm was removed, sometimes even bringing the exumbrella surfaces in contact. While this seemed in no way to interfere with regeneration, it frequently made accurate measurements impossible unless the specimen was first narcotized, as any attempt to unfold the active disk usually resulted in tearing the delicate regenerating tissue. This procedure involving the expenditure of so much time, all badly folded specimens were discarded. If the folding took place some days after the start of any series of experiments the specimen was discarded and the figures for the earlier stages were retained in the record.

The other most common source of difficulty in making the measurements arose on account of the tendency of the edge of the regenerating tissue to fuse with the edge of the old cut surface or with a more proximal part of the sheet of regenerating tissue. Whenever the edge of the thin sheet of new tissue became folded back sufficiently to touch any of the more proximal tissues fusion took place, so that a tube would be formed from the new tissue. When the folding involved only a small

TABLE 1.—*Record of experiment 4a, started September 30, 1913 (for 1 to 5 days).*

[Forty disks each with all sense-organs removed from one side and with the halves insulated by removal of 2 strips of subumbrella ectoderm. The letter C after the measurement for any day indicates that the central cavity had been filled by the regenerated tissue.]

Specimen.	1 day.	2 days.	3 days.	4 days.	5 days.
No. 1 Half with S. O.	3.25	4.75	9.50 C	.....	.....
No. 1 Half without S. O.	2.00	2.50	6.00	.....	.....
No. 2 Half with S. O.	2.50	4.00	5.00	9.00 C	.....
No. 2 Half without S. O.	1.50	3.00	3.75	6.25	.....
No. 3 Half with S. O.	2.25	3.00	5.50	8.25 C	.....
No. 3 Half without S. O.	1.75	2.00	3.25	5.00	.....
No. 4 Half with S. O.	2.75	4.00	5.75	14.75 C	.....
No. 4 Half without S. O.	1.75	3.00	4.50	10.50	.....
No. 5 Half with S. O.	2.00	3.75	5.50	7.25 C	9.00
No. 5 Half without S. O.	1.50	3.00	4.75	6.00	7.50
No. 6 Half with S. O.	1.50	3.00	3.75	5.00 C	6.75 C
No. 6 Half without S. O.	0.80	2.25	2.75	3.25	4.50
No. 7 Half with S. O.	3.75	5.00	5.75	7.50	.....
No. 7 Half without S. O.	1.75	2.00	3.00	5.00	.....
No. 8 Half with S. O.	3.50	4.00	4.00	9.50 C	.....
No. 8 Half without S. O.	2.50	3.00	3.00	7.00	.....
No. 9 Half with S. O.	2.75	3.75	5.00	5.25	6.00 C
No. 9 Half without S. O.	2.25	3.00	3.85	4.00	4.30
No. 10 Half with S. O.	3.25	5.50	8.25	9.50 C	.....
No. 10 Half without S. O.	2.50	4.00	6.50	4.25	.....
No. 11 Half with S. O.	3.00	4.00	6.00	8.00 C	.....
No. 11 Half without S. O.	2.15	3.00	4.00	6.25	.....
No. 12 Half with S. O.	2.50	5.00	6.50	7.50	9.00 C
No. 12 Half without S. O.	1.75	3.75	4.50	5.25	6.50
No. 13 Half with S. O.	1.50	4.00	4.25	6.00	8.75 C
No. 13 Half without S. O.	1.00	3.75	4.00	5.00	7.00
No. 14 Half with S. O.	4.00	5.00	6.80	7.00 C	.....
No. 14 Half without S. O.	3.00	4.00	5.25	5.25	.....
No. 15 Half with S. O.	....	3.00	4.50	7.00	10.00
No. 15 Half without S. O.	....	2.00	3.50	5.50	7.00
No. 16 Half with S. O.	2.50	3.75	4.75	6.00	10.50 C
No. 16 Half without S. O.	1.50	2.50	3.25	4.15	8.00
No. 17 Half with S. O.	2.75	3.75	6.25	7.75 C	.....
No. 17 Half without S. O.	2.50	3.00	5.00	6.00	.....
No. 18 Half with S. O.	3.00	4.00	5.50	8.00 C	.....
No. 18 Half without S. O.	2.00	3.00	3.75	5.75	.....
No. 19 Half with S. O.	2.75	3.50	4.25	6.00 C	.....
No. 19 Half without S. O.	2.75	3.00	3.50	5.00	.....
No. 20 Half with S. O.	2.75	4.00	6.00	9.00	14.00 C
No. 20 Half without S. O.	2.00	2.50	3.75	7.00	9.00
No. 21 Half with S. O.	2.50	3.50	4.50	7.00 C	.....
No. 21 Half without S. O.	1.50	2.75	3.50	5.50	.....
No. 22 Half with S. O.	2.75	3.50	Grown together.		
No. 22 Half without S. O.	1.50	2.50			
No. 23 Half with S. O.	3.00	4.25	5.75	9.00	.....
No. 23 Half without S. O.	2.00	3.00	4.25	8.25	.....
No. 24 Half with S. O.	2.00	4.50	5.50 C	.....	.....
No. 24 Half without S. O.	1.25	2.75	3.50	.....	.....
No. 25 Half with S. O.	2.25	4.75	8.00 C	.....	.....
No. 25 Half without S. O.	1.50	3.15	5.50	.....	.....
No. 26 Half with S. O.	2.50	3.50	5.50	8.25 C	.....
No. 26 Half without S. O.	1.50	2.25	3.75	6.50	.....
No. 27 Half with S. O.	2.00	4.50	6.75 C	.....	.....
No. 27 Half without S. O.	1.50	3.75	5.00	.....	.....
No. 28 Half with S. O.	2.25	4.00	6.00 C	.....	.....
No. 28 Half without S. O.	1.50	3.00	4.50	.....	.....

TABLE 1.—*Record of experiment 4a, started September 30, 1913 (for 1 to 5 days)*—Continued.

[Forty disks each with all sense-organs removed from one side and with the halves insulated by removal of 2 strips of subumbrella ectoderm. The letter C after the measurement for any day indicates that the central cavity had been filled by the regenerated tissue.]

Specimen.	1 day.	2 days.	3 days.	4 days.	5 days.
No. 29 Half with S. O.	2.50	3.75	4.50	Badly wrinkled.	
No. 29 Half without S. O.	2.00	3.00	3.50		
No. 30 Half with S. O.	3.00	3.55	3.75	4.75	7.00 C
No. 30 Half without S. O.	1.20	2.00	2.50	3.25	5.50 C
No. 31 Half with S. O.	2.00	3.00	5.00	7.00 C	.....
No. 31 Half without S. O.	1.00	2.25	3.50	5.00 C	.....
No. 32 Half with S. O.	2.00	3.00	6.50	10.75 C	.....
No. 32 Half without S. O.	1.25	2.00	4.00	7.50 C	.....
No. 33 Half with S. O.	1.75	2.50	4.50	6.00 C	.....
No. 33 Half without S. O.	0.50	1.00	2.50	4.00 C	.....
No. 34 Half with S. O.	2.00	4.00	6.00 C	.....	.....
No. 34 Half without S. O.	1.50	3.00	4.00 C	.....	.....
No. 35 Half with S. O.	3.00	Too badly wrinkled to measure new tissue.			
No. 35 Half without S. O.	2.00				
No. 36 Half with S. O.	3.75	8.25 C	....	.....	.....
No. 36 Half without S. O.	2.00	5.00 C	....	.....	.....
No. 37 Half with S. O.	2.75	4.00	7.00 C	.....	.....
No. 37 Half without S. O.	1.50	3.00	5.50 C	.....	.....
No. 38 Half with S. O.	1.50	2.25	Badly folded.		
No. 38 Half without S. O.	1.00	1.75			
No. 39 Half with S. O.	2.00	5.25	7.50 C	.....	.....
No. 39 Half without S. O.	1.25	3.00	5.25 C	.....	.....
No. 40 Half with S. O.	4.25	6.00 C	....	.....	.....
No. 40 Half without S. O.	3.00	4.75 C	....	.....	.....
Mean of all observations:					
Half with S. O.	2.65	4.05	5.75	7.63	9.00
Half without S. O.	1.73	2.87	4.06	5.57	6.36

area separation could be easily accomplished, but if a considerable portion of the regenerating sheet was involved the specimen was rendered useless for further study.

The results of two typical experiments are shown in table 1. The measurements are in millimeters. The upper figure for each date shows the width of the sheet of tissue regenerated from the active half-disk, the lower figure the width of that regenerated from the inactive half-disk. When the sheet of new tissue had entirely closed over the cavity in the center of the disk the point of closure remained recognizable for at least a day, so that the measurements could readily be made for those disks that had become closed since the time of the last measurements. By the end of 24 hours after the new sheet of tissue was completed the point of closure would be shifted until it came to lie in the center of the disk.

The results from the 40 disks recorded in table 1 are shown in figure 6, in which the divisions along the abscissa show the time of regeneration in days, those along ordinate the amount of regeneration measured in millimeters. The record for each specimen is carried to the time of closure of the open circle in the center of the disk by the sheet of regenerated tissue.

From the start of regeneration the new tissue produced from the side with its sense-organs intact is shown to be more rapid. In the early stage of regeneration this difference is more striking upon a cursory examination than in the later stages, although the actual difference on the rate of growth of new tissue changes only slightly during the entire period of regeneration. The proportion between the amounts of new tissue formed each day, taking the amount regenerated from the side without sense-organs as the unit, was respectively: First day 1:1.53; second day 1:1.44; third day 1:1.41; fourth day 1:1.38; fifth day 1:1.39.

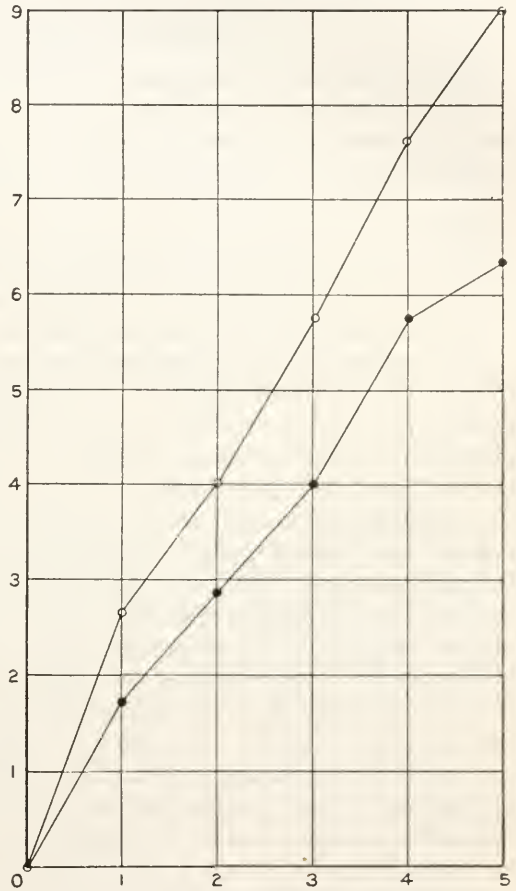

FIG. 6.—Showing relative rates of regeneration of the halves of 40 disks. The upper line represents the half-disks with sense-organs, the lower line represents those without sense-organs.

The regeneration from the half without sense-organs is more regular, as is shown by the fact that for the mean of each day's observation the probable error is less for that half than for the one upon which the sense-organs remain. This result would be expected to follow from the fact that the inactive side was relieved from the influence of the marginal sense-organs, which would introduce many stimuli of varying intensity, all of which would have either a retarding or accelerating influence upon the processes of regeneration.

When the rates of regeneration of certain disks in table 1 are compared with one another, the cause of the uncertainty of the results obtained in experiments with entire disks is clearly shown. The

closure of the open circle in disk 36, table 1, was complete in 2 days, while 5 days were necessary to complete the closure in specimen 30, table 1. Had the latter been the active disk and the former the inactive disk of a pair compared in an experiment with entire disks, the conclusion that an inactive disk sometimes regenerates more rapidly than an active one could not have been avoided.

EXPERIMENTS WITH ANESTHETICS.

In the previously described experiments it was shown that the active half of a medusa disk prepared as shown in figures 3 and 5 has a higher rate of regeneration than does the inactive half of the same specimen. While this point is clearly shown in all the experiments of the two types just mentioned, the results obtained by this method throw no light upon the nature of the control exercised by the marginal sense-organs as to whether it is exerted through the higher metabolism brought about by muscular activity or through some other less apparent metabolic process under the control of the sense-organs.

Two other types of experiments were undertaken to ascertain the nature of the nervous control. In the first set of experiments (type 3) disks prepared with insulated active and inactive halves (fig. 3) were allowed to regenerate in sea-water to which 15 per cent by volume of 0.6 m $MgSO_4$ had been added. In this solution the disks will live for an indefinite time and will for several hours retain the capacity to regain their normal activity within a few moments after being returned to fresh sea-water. Mayer (*op. cit.*) has shown that the effect of the magnesium in a weak $MgSO_4$ solution in sea-water is for a time confined almost entirely to the muscular tissues, while the nervous network is still capable of transmitting the impulse necessary for pulsation over an area submerged in the magnesium solution where no contraction of the muscles could be observed. When kept in the magnesium sea-water for a prolonged period the sense-organs become incapable of giving rise to the stimulus necessary for normal pulsation long before the nervous network loses its capacity for transmitting such a stimulus, so that a ring cut from a medusa disk and activated by a circuit wave of contraction will show by an indicator strip in sea-water (Mayer, *loc. cit.*, page 122) the transmission of the nervous impulse for some time after a ring retaining its sense-organs is no longer able to activate its indicator strip.

When medusa disks prepared with insulated active and inactive halves are put into the magnesium sea-water they lose their power of muscular movement within a few moments. Usually all of the disks float on the surface of the new solution for 20 to 30 minutes before they become adjusted to the abnormally dense medium; at the end of this period they settle to the bottom of the jar and remain completely relaxed throughout the experiment.

During the first 12 hours of an experiment, or as soon as the newly regenerated tissue became recognizable, regeneration is more rapid from the side on which the sense-organs are present. From that time on, the regeneration from the two halves is (within the limits of error of the measurements) about equal. The rate of regeneration of the half-disk with sense-organs fails to equal that of the half-disk without sense-organs. For both halves the rate was noticeably lower than that of the inactive half of a disk in normal sea-water. The lack of proper aeration commonly brought about through the pulsation of the active half-disk may account in part for the lower rate of regeneration, but there is unquestionably some more fundamental disturbance in the metabolic activity caused by the presence of the excess of Mg ions in the fluid.

The results from experiments of this type with the halves of 40 disks is given in table 2 and is shown graphically in figure 7.

TABLE 2.—*Record of experiments 12 and 12a (for 1 day to 9 days).*

[Forty disks each with insulated halves, one with sense-organs, the other without. Mg sea-water. The letter C after the measurement for any day indicates that the central cavity had been filled by the regenerated tissue.]

Specimen.	1 day.	2 days.	3 days.	4 days.	5 days.	6 days.	7 days.	8 days.	9 days.
No. 1 Half with S. O....	2.00	2.50	3.25	3.75	4.50	5.25	6.00	6.60	6.40
No. 1 Half without S. O.	0.80	1.40	2.00	2.75	3.40	4.20	4.80	5.70	6.55
No. 2 Half with S. O....	1.75	2.30	2.90	3.75	4.25	5.00	6.00	6.50	7.25
No. 2 Half without S. O.	0.90	1.50	1.75	2.50	3.25	4.00	4.75	5.25	6.00
No. 3 Half with S. O....	1.00	1.50	2.75	3.75	4.50	5.00	5.75	6.50	7.00
No. 3 Half without S. O.	0.50	0.75	1.25	2.00	3.00	3.75	4.50	5.25	6.25
No. 4 Half with S. O....	1.50	2.25	3.00	3.50	4.25	5.00	5.75	6.50	7.25
No. 4 Half without S. O.	1.00	1.50	2.00	2.75	3.25	3.75	4.50	5.25	6.00
No. 5 Half with S. O....	1.75	2.25	3.00	3.75	4.50	5.25	5.75	6.25	7.00
No. 5 Half without S. O.	1.00	1.50	2.25	2.00	3.50	4.00	4.50	5.00	5.75
No. 6 Half with S. O....	2.00	2.75	3.50	4.25	5.25	5.75	6.25	7.00	C
No. 6 Half without S. O.	1.25	1.75	2.50	3.00	3.75	4.25	4.75	5.75	
No. 7 Half with S. O....	1.00	2.00	3.00	3.75	4.75	5.50	6.25	7.00	7.50
No. 7 Half without S. O.	0.75	1.25	2.00	2.50	3.25	4.00	4.50	5.25	5.75
No. 8 Half with S. O....	1.25	1.50	2.25	3.00	4.00	4.75	5.50	6.25	7.00
No. 8 Half without S. O.	0.75	1.00	1.25	1.75	3.50	3.25	3.75	4.50	5.25
No. 9 Half with S. O....	2.25	2.75	3.50	4.00	4.75	5.50	6.25	7.25	C
No. 9 Half without S. O.	1.00	1.50	2.25	2.75	3.25	4.00	4.50	5.25	
No. 10 Half with S. O....	1.50	2.25	3.00	3.50	4.25	5.00	5.50	6.25	7.25
No. 10 Half without S. O.	0.50	1.00	1.75	2.25	3.00	3.75	4.50	5.25	6.00
No. 11 Half with S. O....	1.00	1.75	2.50	3.50	4.25	5.00	5.75	6.50	7.25
No. 11 Half without S. O.	0.50	1.00	1.75	2.50	3.25	4.00	4.50	5.25	6.00
No. 12 Half with S. O....	2.50	3.25	4.00	4.75	5.50	6.25	7.00	7.75	C
No. 12 Half without S. O.	1.25	1.75	2.25	2.75	3.25	4.00	4.50	5.25	
No. 13 Half with S. O....	1.75	2.25	3.00	3.75	4.50	5.25	5.75	6.50	7.25
No. 13 Half without S. O.	0.50	1.25	1.75	2.50	3.25	4.00	4.75	5.50	6.00
No. 14 Half with S. O....	0.75	1.50	2.25	2.75	3.75	4.50	5.25	6.25	7.25
No. 14 Half without S. O.	0.25	0.75	1.50	2.25	3.00	3.75	4.50	5.25	6.25
No. 15 Half with S. O....	1.50	2.00	2.75	3.75	4.50	5.25	6.00	6.75	7.25
No. 15 Half without S. O.	0.50	1.25	1.75	2.50	3.25	4.00	4.75	5.25	6.00
No. 16 Half with S. O....	2.00	2.75	3.25	4.00	4.50	5.25	6.00	6.50	7.25
No. 16 Half without S. O.	0.75	1.25	1.75	2.50	3.25	4.00	4.75	5.25	6.00

TABLE 2.—*Record of experiments 12 and 12a (for 1 day to 9 days)*—Continued.

[Forty disks each with insulated halves, one with sense-organs, the other without. Mg. sea-water. The letter C after the measurement for any day indicates that the central cavity had been filled by the regenerated tissue.]

Specimen.	1 day.	2 days.	3 days.	4 days.	5 days.	6 days.	7 days.	8 days.	9 days.
No. 17 Half with S. O....	1.75	2.50	3.25	4.00	4.75	5.25	6.00	6.75	7.50
No. 17 Half without S. O	0.50	1.25	2.00	2.50	3.25	4.00	4.75	5.25	6.00
No. 18 Half with S. O....	2.25	3.00	3.75	4.50	5.00	6.00	7.00	C	....
No. 18 Half without S. O.	1.00	1.50	2.25	2.75	3.50	4.25	5.25		....
No. 19 Half with S. O....	2.75	3.25	4.50	5.25	6.00	6.75	7.50	C	....
No. 19 Half without S. O.	1.50	2.00	2.50	3.25	3.75	4.25	5.00		....
No. 20 Half with S. O....	1.50	2.25	3.00	3.75	4.50	5.50	6.25	7.00	7.75
No. 20 Half without S. O.	0.75	1.50	2.00	2.50	3.25	4.00	4.75	5.25	6.00
No. 21 Half with S. O....	3.00	3.50	4.25	4.75	5.25	6.00	6.75	7.50	C
No. 21 Half without S. O.	1.50	2.00	2.50	3.25	3.75	4.50	5.25	6.00	
No. 22 Half with S. O....	2.25	2.75	3.25	3.75	4.50	5.25	6.00	6.50	7.25
No. 22 Half without S. O.	1.00	1.50	2.00	2.50	3.25	4.00	4.75	5.50	6.25
No. 23 Half with S. O....	1.50	2.25	3.00	3.75	4.25	5.00	5.75	6.50	7.25
No. 23 Half without S. O.	0.75	1.25	2.00	2.75	3.50	4.25	5.00	5.75	6.00
No. 24 Half with S. O....	2.00	2.50	3.25	3.75	4.50	5.00	5.75	6.25	7.00
No. 24 Half without S. O.	0.75	1.50	2.25	1.75	3.25	3.75	4.50	5.00	5.75
No. 25 Half with S. O....	1.25	2.00	2.75	3.50	4.75	5.25	6.00	6.75	7.50
No. 25 Half without S. O.	0.50	1.00	1.50	2.25	2.00	3.50	4.25	5.00	6.00
No. 26 Half with S. O....	1.75	2.25	3.00	4.00	4.75	5.25	6.00	7.00	8.00
No. 26 Half without S. O.	0.50	1.00	1.50	2.50	3.25	3.75	4.50	5.25	6.25
No. 27 Half with S. O....	2.50	3.25	4.00	4.75	5.50	6.50	7.50	8.25	C
No. 27 Half without S. O.	1.00	1.75	2.50	3.25	4.00	4.75	5.50	6.25	
No. 28 Half with S. O....	2.25	3.00	3.50	4.00	4.75	5.50	6.25	7.00	7.75
No. 28 Half without S. O.	1.00	1.75	2.25	3.00	3.50	4.00	4.75	5.25	6.00
No. 29 Half with S. O....	1.75	2.50	2.00	3.75	4.25	5.00	5.75	6.50	7.25
No. 29 Half without S. O.	1.00	1.75	2.25	2.75	3.50	4.25	5.00	5.75	6.50
No. 30 Half with S. O....	2.75	3.25	4.00	5.00	6.00	6.75	7.50	C	....
No. 30 Half without S. O.	1.75	1.50	2.25	3.00	3.75	4.00	5.00		....
No. 31 Half with S. O....	2.25	3.00	3.75	4.50	5.25	6.00	6.75	7.50	C
No. 31 Half without S. O.	1.00	1.50	2.25	3.00	3.75	4.50	5.25	6.00	
No. 32 Half with S. O....	1.50	2.50	3.25	3.75	4.50	5.25	6.00	6.75	7.50
No. 32 Half without S. O.	0.50	1.25	2.00	2.75	3.50	4.25	5.00	5.75	6.25
No. 33 Half with S. O....	2.00	2.75	3.25	4.00	4.50	5.25	6.00	6.75	7.50
No. 33 Half without S. O	0.75	1.50	2.00	2.75	3.25	4.00	4.75	5.50	6.25
No. 34 Half with S. O....	1.00	1.75	2.50	3.25	4.00	5.00	5.75	6.50	7.25
No. 34 Half without S. O.	0.25	1.00	1.75	2.00	2.75	3.50	4.25	5.00	5.75
No. 35 Half with S. O....	1.75	2.50	3.25	3.75	4.50	5.26	6.00	6.75	7.50
No. 35 Half without S. O.	1.00	1.50	2.25	2.75	3.50	4.25	4.75	5.50	6.25
No. 36 Half with S. O....	2.25	3.00	3.50	4.00	4.50	5.25	6.00	6.50	7.25
No. 36 Half without S. O	1.00	1.75	2.50	3.25	4.00	4.75	5.50	6.00	6.75
No. 37 Half with S. O....	1.50	2.25	3.00	3.75	4.50	5.25	6.00	6.75	7.75
No. 37 Half without S. O.	0.75	1.50	2.25	2.75	3.50	4.25	5.00	5.75	6.50
No. 38 Half with S. O....	2.75	3.50	4.25	5.00	5.75	6.50	7.25	8.00	C
No. 38 Half without S. O.	1.00	1.75	2.50	3.25	4.00	4.75	5.50	6.00	
No. 39 Half with S. O....	2.25	3.00	3.50	4.25	5.00	5.50	6.00	6.75	7.50
No. 39 Half without S. O.	0.75	1.50	2.25	3.00	3.75	4.50	5.00	5.75	6.50
No. 40 Half with S. O....	1.50	2.25	3.00	3.50	4.25	5.00	5.75	6.50	7.25
No. 40 Half without S. O.	0.50	1.25	2.75	2.75	3.50	4.25	5.00	5.75	6.50
Mean of all observations:									
Half with S. O....	2.00	2.35	2.92	3.55	4.57	5.17	5.94	6.47	7.40
Half without S. O.	0.85	1.43	2.00	2.85	3.45	4.23	4.87	5.74	6.47

Experiments with KCN or chloroform dissolved in sea-water did not give satisfactory results. In both these solutions the tissues of the medusa underwent rapid disintegration if the amount of the reagent present was sufficient to bring about any noticeable effect upon the activity of the sense-organs.

When medusæ are treated with a weak solution of oxalic acid in magnesium-free artificial sea-water it is possible to destroy the activity of the sense-organs for a considerable time without seriously injuring the other tissues. In all my experiments, however, recovery of functional activity by the sense-organs took place within 24 hours if the oxalic-acid solution was of such a strength that the ectodermal tissues were not injured. In such experiments there was at first an equal rate of regeneration for each of the halves of a disk until the sense-organs

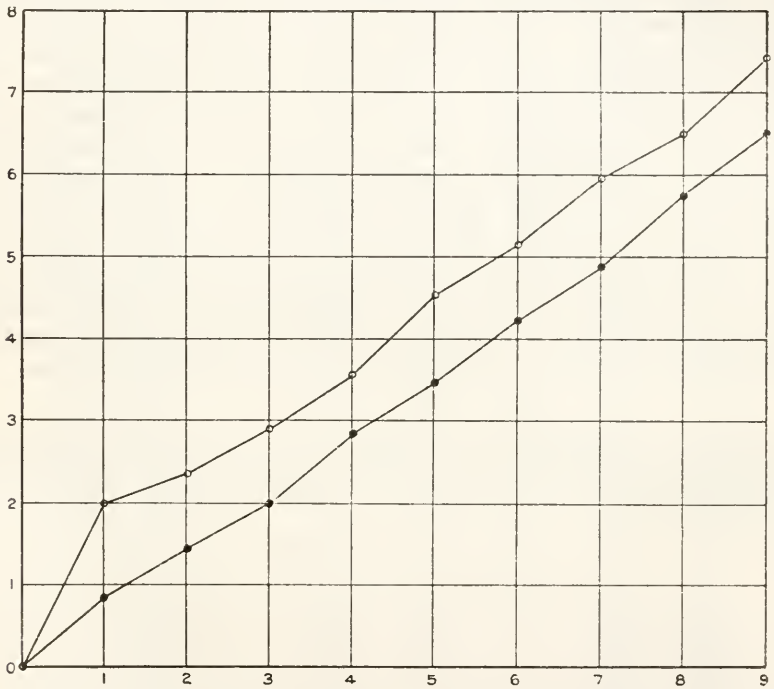

Fig. 7.—Showing the rates of regeneration of active and inactive half-disks regenerating in sea-water to which has been added 15 parts of 0.6 m $MgSO_4$. The upper line represents the half with sense-organs; the lower line represents those without sense-organs. The divisions along the ordinate represent the amount of regeneration in millimeters; those along the abscissa the time of regeneration in days.

regained their functional activity, after which the half-disk with sense-organs regenerated most rapidly.

The experiments with Mg solutions show that there is an influence of the sense-organs on the rate of regeneration which is apparently exercised for a considerable time after muscular activity has been suppressed. It was impossible, however, by this method to differentiate with any certainty between the two effects, since there is no visual means of ascertaining the exact time at which the sense-organs lose their power of sending out the stimuli necessary for normal contraction. Since, as was shown by Mayer (*op. cit.*), it is possible to maintain a

circuit wave of contraction in the muscles of a half-disk without sense-organs by making a series of cuts by which an endless labyrinth of subumbrella tissue is formed (figs. 4 and 5), the part played by muscular activity and that by the stimuli from the sense-organs can be directly compared. In such experiments all the sense-organs were removed from the medusa disks, the halves insulated, and a circuit wave of contraction started by an induction shock in a labyrinth cut in the muscle-tissues of one of its halves. Once established, the contraction wave would be maintained throughout the course of an experiment unless interrupted by an unusually strong stimulus through some accident in handling. When interrupted in this way the circuit wave could be established again by renewed electrical stimulation. The amplitude of the contraction wave becomes gradually reduced as time goes on, but there is little variation in its rate. When the rates of regeneration of the halves of any disk prepared in this manner are compared, it is found that the half in which the circuit wave is maintained regenerates slightly faster than the inactive one. This difference in rate is, however, very much less than between the halves of a disk from one-half of which the sense-organs have been removed (compare figs. 6 and 8), although the activated disk pulsates from 3 to 10 times as fast as the one under the control of the sense-organs.

TABLE 3.—*Record for 1 to 9 days.*

[Forty specimens from which all of the sense-organs had been removed and the subumbrella muscles of one half activated by a circuit wave of contraction (fig. 8). The letter C after the measurement for any day indicates that the central cavity had been filled by the regenerated tissue.]

Specimen.	1 day.	2 days.	3 days.	4 days.	5 days.	6 days.
No. 1 Activated half	2.00	3.25	4.50	6.25	7.25	8.00
No. 1 Half without S. O	1.75	2.75	4.00	5.50	6.50	7.25
No. 2 Activated half	2.50	3.25	4.75	6.50	8.00	C
No. 2 Half without S. O	2.00	3.00	4.25	6.00	7.50	
No. 3 Activated half	1.75	3.00	4.50	6.00	7.00	8.25
No. 3 Half without S. O	1.50	2.25	3.25	5.25	6.25	7.00
No. 4 Activated half	1.50	2.75	4.00	6.00	7.25	8.25
No. 4 Half without S. O	1.25	2.50	3.50	5.25	6.00	7.00
No. 5 Activated half	2.25	3.25	4.50	6.25	7.00	8.25
No. 5 Half without S. O	1.75	2.75	4.00	5.50	6.25	7.25
No. 6 Activated half	2.00	3.25	4.75	6.00	7.50	8.50
No. 6 Half without S. O	1.75	2.75	4.00	5.50	6.50	7.25
No. 7 Activated half	2.75	4.00	5.00	6.50	8.00	C
No. 7 Half without S. O	2.25	3.25	4.25	5.50	7.00	
No. 8 Activated half	3.00	4.00	5.25	6.50	8.00	C
No. 8 Half without S. O	2.50	3.00	4.50	5.75	7.25	
No. 9 Activated half	1.00	2.25	3.50	5.25	6.50	8.00
No. 9 Half without S. O	0.75	1.75	3.00	3.75	5.75	7.25
No. 10 Activated half	1.75	3.00	4.25	6.00	7.50	8.25
No. 10 Half without S. O	1.50	2.50	3.50	5.00	6.25	7.00
No. 11 Activated half	2.50	3.25	4.50	6.25	7.25	8.00
No. 11 Half without S. O	2.25	2.75	4.00	3.75	6.75	7.50
No. 12 Activated half	3.50	4.25	5.25	6.75	8.25	C
No. 12 Half without S. O	3.25	3.75	4.75	6.00	7.50	

TABLE 3.—*Record for 1 to 9 days*—Continued.

Forty specimens from which all of the sense-organs had been removed, and the subumbrella muscles of one half activated by a circuit wave of contraction (fig. 8). The letter C after the measurement for any day indicates that the central cavity had been filled by the regenerated tissue.]

Specimen.	1 day.	2 days.	3 days.	4 days.	5 days.	6 days.
No. 13 Activated half	1.25	2.50	4.25	6.00	7.50	8.50
No. 13 Half without S. O	1.00	2.25	4.00	5.50	6.75	7.25
No. 14 Activated half	2.00	3.25	4.50	6.25	7.25	8.25
No. 14 Half without S. O	1.75	2.75	4.00	5.75	6.50	7.25
No. 15 Activated half	2.25	3.50	4.50	6.50	8.00	C
No. 15 Half without S. O	2.00	3.00	4.00	6.00	7.25	
No. 16 Activated half	1.00	2.50	4.25	6.00	7.25	8.25
No. 16 Half without S. O	0.75	2.25	4.00	5.00	6.25	7.00
No. 17 Activated half	1.75	3.00	4.25	6.00	7.25	8.00
No. 17 Half without S. O	1.50	2.50	3.75	5.50	6.75	7.50
No. 18 Activated half	2.50	3.50	4.50	6.50	8.50	C
No. 18 Half without S. O	2.25	3.00	4.00	6.00	7.75	
No. 19 Activated half	1.25	2.75	4.00	6.00	7.00	7.75
No. 19 Half without S. O	1.00	2.50	3.75	5.75	6.75	7.50
No. 20 Activated half	2.00	3.25	4.50	6.00	7.25	8.00
No. 20 Half without S. O	1.75	2.75	4.00	5.75	6.50	7.25
No. 21 Activated half	1.75	3.00	4.50	6.25	7.25	8.00
No. 21 Half without S. O	1.50	2.75	4.00	5.50	6.50	7.25
No. 22 Activated half	2.50	3.25	4.50	6.00	7.00	8.25
No. 22 Half without S. O	2.00	2.75	4.00	5.50	6.25	7.25
No. 23 Activated half	2.00	3.00	4.50	6.00	7.00	8.00
No. 23 Half without S. O	1.75	2.75	4.00	5.25	6.25	7.25
No. 24 Activated half	3.00	3.75	5.00	6.25	8.00	C
No. 24 Half without S. O	2.50	3.25	4.25	5.50	7.25	
No. 25 Activated half	1.50	2.50	4.25	5.75	7.00	8.00
No. 25 Half without S. O	1.25	2.25	4.00	5.50	6.50	7.50
No. 26 Activated half	1.00	2.50	4.50	6.25	7.00	8.00
No. 26 Half without S. O	0.75	2.25	4.00	5.50	6.25	7.25
No. 27 Activated half	2.00	3.25	4.50	6.50	7.25	8.00
No. 27 Half without S. O	1.75	3.00	3.75	5.75	6.50	7.25
No. 28 Activated half	2.25	3.25	4.50	6.25	7.00	8.00
No. 28 Half without S. O	1.75	2.75	4.00	5.50	6.25	7.25
No. 29 Activated half	2.00	3.00	4.75	6.25	7.25	8.00
No. 29 Half without S. O	1.75	2.75	4.00	5.50	6.25	7.00
No. 30 Activated half	1.50	2.50	4.00	6.00	7.00	8.25
No. 30 Half without S. O	1.25	2.25	3.75	5.50	6.50	7.25
No. 31 Activated half	2.00	3.00	4.50	6.00	7.25	8.00
No. 31 Half without S. O	1.50	2.50	4.00	5.25	6.25	7.00
No. 32 Activated half	2.75	3.50	4.75	6.75	7.50	8.50
No. 32 Half without S. O	2.25	3.00	4.00	5.75	6.75	7.25
No. 33 Activated half	4.00	5.75	7.75	8.75	C	....
No. 33 Half without S. O	3.50	4.75	7.00	7.75		....
No. 34 Activated half	2.00	3.50	5.00	6.50	8.00	C
No. 34 Half without S. O	1.75	3.00	4.25	5.75	7.50	
No. 35 Activated half	1.25	2.50	4.00	6.00	7.50	8.50
No. 35 Half without S. O	1.00	2.25	3.75	5.75	6.50	7.25
No. 36 Activated half	1.00	2.50	4.25	6.00	7.25	8.00
No. 36 Half without S. O	0.75	2.00	3.75	5.50	6.75	7.50
No. 37 Activated half	1.50	3.00	4.75	6.25	7.25	8.00
No. 37 Half without S. O	1.25	2.75	4.25	5.50	6.50	7.25
No. 38 Activated half	2.00	3.00	4.25	6.00	7.25	8.25
No. 38 Half without S. O	1.75	2.75	4.00	5.50	6.50	7.25
No. 39 Activated half	1.00	2.25	4.00	6.00	7.00	8.25
No. 39 Half without S. O	0.75	2.00	3.75	5.25	6.25	7.50
No. 40 Activated half	2.00	3.00	4.50	6.50	7.50	8.50
No. 40 Half without S. O	1.75	2.50	4.00	5.50	6.25	7.00
Mean of all observations:						
Activated half	2.00	3.27	4.67	6.15	7.23	8.12
Half without S. O	1.73	2.87	4.06	5.57	6.36	7.27

The amount of activity and metabolism in the muscles if they have any noticeable influence on the rate of regeneration ought to produce a clearly demonstrable result, but as shown by the data in table 3 and figure 8 the difference is relatively small. From the point of view of the chemical nature of metabolism (including regeneration) the

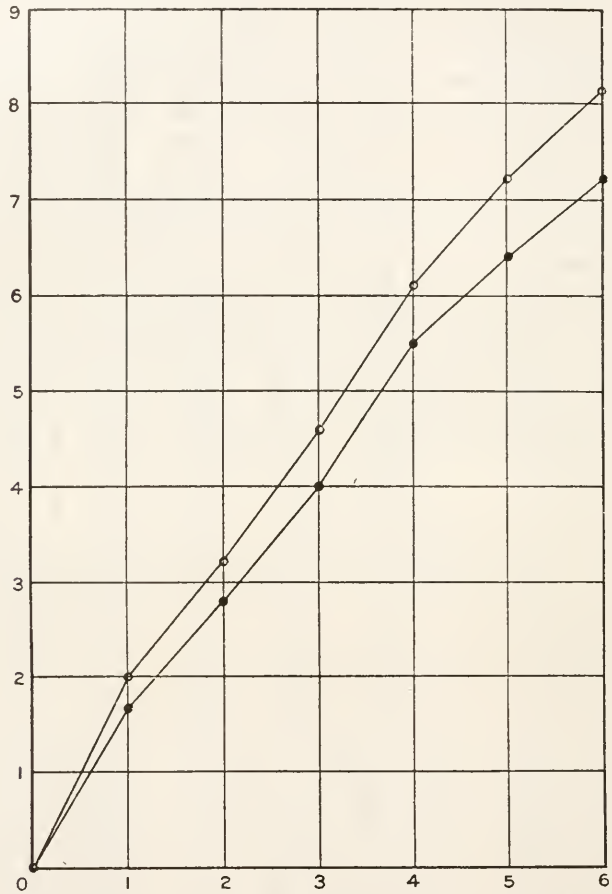

FIG. 8.—Showing rates of regeneration of activated and inactive half-disks. The upper line represents the activated and the lower line the inactive specimens. The divisions along the abscissa represent regeneration in millimeters; those along the ordinate represent time in days.

difference in temperature might conceivably be sufficient to account for the observed difference in rate of regeneration. The half-disks in which the circuit wave is maintained show a greater regularity in the rate of regeneration than do the active disks recorded in table 1.

Further demonstration of the influence of the sense-organs on the rate of regeneration is furnished in another series of experiments in which the two insulated halves of a disk are compared, one of which is

contracting normally under the influence of its sense-organs, while all the sense-organs are removed from the other half and a circuit wave of contraction maintained in a labyrinth of its subumbrella muscles. In a disk prepared in this manner the rate of pulsation of the activated half will vary from 3 to 10 times that of the normally contracting half.

The comparative rates of regeneration for 40 disks under these conditions are shown in table 4 and figure 9.

TABLE 4.—*Record for 1 to 5 days.*

[Forty specimens, one half of each one pulsating under the control of its sense-organs, while a circuit wave of contraction was maintained in the subumbrella muscles of the other half (fig. 10). The letter C after the measurement for any day indicates that the central cavity had been filled by the regenerated tissue.]

Specimen.		1 day.	2 days.	3 days.	4 days.	5 days.
No. 1	Half with S. O.	3.00	4.25	5.75	7.50	9.00
	Activated half.	2.25	3.25	4.50	6.00	7.50
No. 2	Half with S. O.	1.75	3.50	5.50	7.25	9.00
	Activated half.	1.50	3.25	5.25	7.00	8.00
No. 3	Half with S. O.	4.00	5.50	6.75	8.25	C
	Activated half.	3.50	4.75	5.50	7.50	
No. 4	Half with S. O.	2.50	4.25	5.50	7.50	9.50
	Activated half.	2.00	3.75	4.75	6.75	8.00
No. 5	Half with S. O.	2.00	4.00	5.50	7.50	9.00
	Activated half.	1.50	3.25	4.50	6.00	7.50
No. 6	Half with S. O.	2.75	4.25	5.75	7.75	9.25
	Activated half.	2.00	3.50	4.75	6.50	7.50
No. 7	Half with S. O.	2.50	4.00	5.50	7.25	8.75
	Activated half.	2.00	3.25	4.75	6.25	7.75
No. 8	Half with S. O.	2.25	4.25	5.75	7.50	9.00
	Activated half.	1.75	3.00	4.25	6.00	7.50
No. 9	Half with S. O.	3.00	4.50	5.75	7.75	9.25
	Activated half.	2.50	3.75	5.00	6.50	7.50
No. 10	Half with S. O.	1.50	3.00	5.00	7.00	9.00
	Activated half.	1.25	2.75	4.50	6.25	8.00
No. 11	Half with S. O.	2.25	4.00	5.50	7.00	9.00
	Activated half.	1.75	3.25	4.75	6.25	7.50
No. 12	Half with S. O.	3.00	4.75	5.50	7.75	9.25
	Activated half.	2.25	3.25	4.50	6.00	7.25
No. 13	Half with S. O.	2.50	4.00	5.75	7.25	9.00
	Activated half.	2.00	3.50	4.75	6.25	7.25
No. 14	Half with S. O.	2.75	4.25	5.75	7.75	9.25
	Activated half.	2.25	3.50	4.50	6.50	7.75
No. 15	Half with S. O.	3.00	4.25	5.75	8.00	9.25
	Activated half.	2.25	3.25	4.75	6.25	7.50
No. 16	Half with S. O.	4.00	6.00	7.75	9.50	C
	Activated half.	3.25	5.25	7.00	8.25	
No. 17	Half with S. O.	2.50	4.25	5.50	7.25	9.00
	Activated half.	2.00	3.25	4.75	6.50	7.75
No. 18	Half with S. O.	2.00	4.00	5.75	7.25	9.25
	Activated half.	1.50	3.50	4.75	6.00	7.50
No. 19	Half with S. O.	2.75	4.25	5.50	7.25	8.75
	Activated half.	2.00	3.25	4.50	6.50	8.00
No. 20	Half with S. O.	2.25	4.00	5.75	7.50	9.00
	Activated half.	1.50	3.25	4.75	6.25	7.25
No. 21	Half with S. O.	3.00	4.50	6.00	9.00	C
	Activated half.	2.50	4.00	4.75	7.50	
No. 22	Half with S. O.	2.25	4.00	6.00	8.00	C
	Activated half.	2.00	3.75	5.50	7.75	

TABLE 4.—*Record for 1 to 5 days*—Continued.

[Forty specimens, one half of each one pulsating under the control of its sense-organs, while a circuit wave of contraction was maintained in the subumbrella muscles of the other half (fig. 10). The letter C after the measurement for any day indicates that the central cavity had been filled by the regenerated tissue.]

Specimen.	1 day.	2 days.	3 days.	4 days.	5 days.
No. 23 Half with S. O.	2.75	4.25	6.00	7.50	9.00
No. 23 Activated half.	2.25	3.50	5.00	6.25	7.50
No. 24 Half with S. O.	2.50	4.00	5.75	7.75	9.25
No. 24 Activated half.	2.00	3.25	4.75	6.25	7.50
No. 25 Half with S. O.	1.25	3.50	5.50	7.25	9.00
No. 25 Activated half.	1.00	3.00	4.75	6.00	7.00
No. 26 Half with S. O.	3.00	5.00	6.50	9.00	C
No. 26 Activated half.	2.25	3.50	5.00	7.50	
No. 27 Half with S. O.	4.00	6.00	9.00	C	....
No. 27 Activated half.	3.25	5.50	8.25		....
No. 28 Half with S. O.	2.75	4.00	5.75	8.00	9.50
No. 28 Activated half.	2.00	3.25	4.75	6.50	8.00
No. 29 Half with S. O.	1.75	3.75	5.50	7.25	8.50
No. 29 Activated half.	1.00	3.25	4.25	6.00	7.00
No. 30 Half with S. O.	2.50	4.00	5.75	7.75	9.00
No. 30 Activated half.	2.00	3.00	4.75	6.50	7.25
No. 31 Half with S. O.	1.50	3.50	5.50	7.50	8.75
No. 31 Activated half.	1.00	3.00	4.75	6.25	7.25
No. 32 Half with S. O.	3.00	4.25	5.75	7.50	8.50
No. 32 Activated half.	2.25	3.50	4.75	6.25	7.50
No. 33 Half with S. O.	2.25	3.75	5.50	7.50	9.00
No. 33 Activated half.	1.75	3.00	4.25	5.75	7.25
No. 34 Half with S. O.	2.00	3.50	5.50	7.75	9.00
No. 34 Activated half.	1.75	2.75	4.75	6.25	7.00
No. 35 Half with S. O.	2.75	4.00	5.50	7.75	9.25
No. 35 Activated half.	2.00	3.25	4.75	6.25	7.50
No. 36 Half with S. O.	3.00	4.00	5.75	8.00	9.25
No. 36 Activated half.	2.25	3.25	4.75	6.50	7.00
No. 37 Half with S. O.	3.50	5.00	7.25	9.25	C
No. 37 Activated half.	2.25	3.50	5.00	7.50	
No. 38 Half with S. O.	2.75	4.00	5.75	7.75	9.25
No. 38 Activated half.	2.00	3.25	4.50	6.25	7.00
No. 39 Half with S. O.	2.00	3.75	5.50	7.75	8.75
No. 39 Activated half.	1.50	3.25	4.75	6.25	7.25
No. 40 Half with S. O.	4.00	6.00	8.75	C	....
No. 40 Activated half.	2.75	4.50	7.50		....
Mean for all observations:					
Half with S. O.	2.65	4.05	5.75	7.63	9.00
Activated half.	2.00	3.27	4.67	6.15	7.23

As was pointed out previously, the stimulus for pulsation originates, at any given time, from a single sense-organ, and pulsation will be continued at nearly its normal rate by a medusa upon which a single sense-organ remains. To test the efficacy of a single sense-organ to control activities other than muscular contraction a series of disks prepared as above described (except that each "active" half-disk had a single sense-organ) were used in a parallel series of regeneration experiments. In all instances such active disks showed an unusual irregularity in the rate of pulsation, since there was present no other nerve-center to take up the initiation of impulses causing pulsation whenever the rate of discharge of the single organ fell below normal.

There was noticeable an indefinite sort of rhythm in the rate of pulsation, as if the sense-organ underwent periods of fatigue from which it was enabled to recover after having given off the stimuli for pulsation for a time at a rate considerably lower than that observed previous to the removal of the other centers. Those periods of depression in the pulsation-rate were of such short duration that they could not be distinguished in the amount of tissue regenerated, if, as would seem probable, the sense-organs were affecting other metabolic activities in a similar manner.

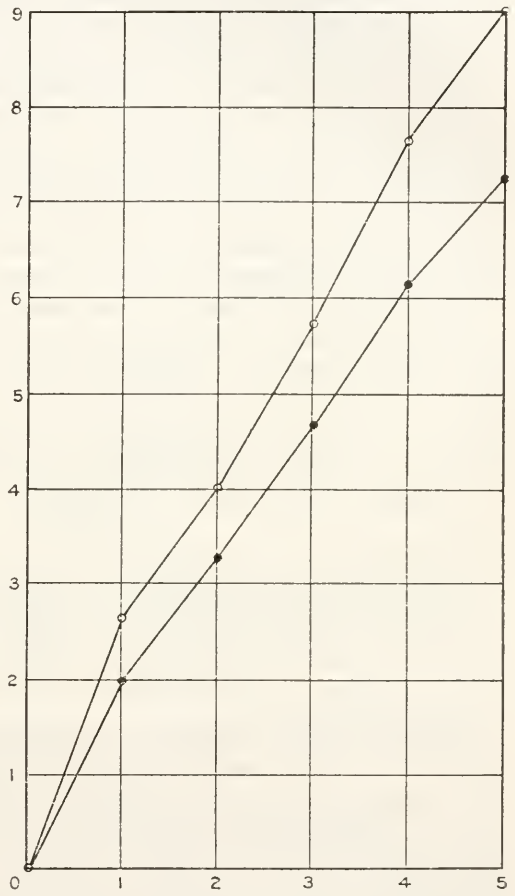

FIG. 9.—Showing rates of regeneration of active and activated half-disks. The divisions along the ordinate represent regeneration in millimeters; those along the abscissa represent time in days.

In all experiments of this type the results were of the same nature as in those when the sense-organs on the activated half-disks had not been disturbed. The amount of time necessary to bring about the closing of the cavity in which regeneration was measured was not sufficiently different from that in specimens where the active half-disk retained its sense-organs to indicate any serious disturbance in metabolic activity. The different specimens in these, as in the previous series, showed great variations in physiological activity (see tables 1, 2, 3, and 4), but there was nothing strikingly characteristic about the behavior of the specimens with a single sense-organ.

EFFECT OF TIME INTERVAL BETWEEN SEPARATION OF DISKS INTO HALVES AND REMOVAL OF SENSE-ORGANS.

In all the experiments on regeneration involving half-disks which retained their sense-organs it was noticeable that the difference in rate of regeneration from the two halves was most marked during

the first day (see figures 6, 7, 8, 9). After that time the rates of the two halves gradually became more nearly equal, until at the end of an experiment, if the amount to be regenerated was large, there was only a slight difference between the rates of the two halves.

In a series of experiments designed to test the importance of the time factor in the difference in the rate of regeneration, sets of 10 disks each were separated into halves and the sense-organs were removed from one of the halves of each pair at intervals of 2 hours. It was soon found that no effect was noticeable if the centers were removed in less than 18 hours, and in most experiments 25 or more hours were necessary in order that the rate of regeneration should be the same from each half of a disk. When the second operation was made in less than 24 hours from the time of dividing the disk it was impossible to distinguish between such specimens and the controls from which half of the sense-organs were removed at the time of the first operation.

Table 5 shows the effect of the removal of the sense-organs from a half-disk of a pair at different intervals after the separation of the disks into halves, each series consisting of 10 specimens.

TABLE 5.—*Effect upon regeneration of the time interval between the separation of a disk into halves and the removal of the sense-organs from one half.*

Series.	First operation.	Rhopalia removed.	Results.
No. 1...	$9^h\ 45^m$ a. m. July 22	7 a. m. July 23	Half with sense-organ fastest.
No. 2...	$8^h\ 00^m$ a. m. July 24	7 p. m. July 24	Half with sense-organ fastest.
No. 3...	$7^h\ 15^m$ a. m. July 25	8 a. m. July 26	Regeneration equal.
No. 4...	$8^h\ 00^m$ a. m. July 25	4 p. m. July 25	Half with sense-organ fastest.
No. 5...	$7^h\ 00^m$ a. m. July 26	8 a. m. July 27	Regeneration equal.

As shown above, the line of demarcation between the time when no influence of the sense-organs will be appreciable and that after which their removal has no effect is sharply drawn at an interval of about 25 hours. In each series of disks there was observed the same irregularity in physiological activity that was shown among the members of any large series. The individuals of the highest physiological activity, as shown in the rate of regeneration, were the first to show the effects of the sense-organs. In any series those specimens from which the sense-organs could first be removed with the assurance that their influence would be shown on the later stages of regeneration could be distinguished after 12 hours by observing the rate at which regeneration was taking place.

The foregoing data seem to show conclusively that there is a clearly marked influence of the sense-organs upon the rate of regeneration in *Cassiopea*. There is no evidence, however, that the presence of this

influence is necessary for the formation of normal structures in regeneration, as Herbst (1896 and 1899) concluded from his experiments on decapods; or as maintained by several of the earlier workers upon regeneration in platodes, and by Walter (1911) for *Triton*. The second of these special cases has been shown by the studies of Morgan, Child, and Goldfarb to be conditioned by influences other than the presence of the nerve-centers. The work of Steele has shown also that in several species of crustacea the removal of an eye-stalk is not followed by the regeneration of a heteromorphic structure.

While none of these workers has laid any stress on the fact that the nervous system exerts an influence on the rapidity of the early stages of regeneration, it has been noted in several instances that the initial stages of regeneration are more rapid in the control animals than in those from which the nervous system has been removed. Thus, Goldfarb (*op. cit.*, page 664) states that in salamanders the hind limb develops more slowly on the side from which the dorsal ganglia innervating the leg had been removed than does that of the opposite side in which the ganglia remained when the spinal cord had already been removed. In a table on pages 665 and 666 he shows that this result was observed in all the specimens recorded save two, in one of which the regeneration was, at the time of measurement, equal for both legs, while in a single specimen the regeneration was most rapid from the side from which the ganglia were removed.

In tadpoles from which the caudal portion of the spinal cord had been removed regeneration of the tail took place more slowly than in the control animals in which the cord was uninjured (p. 672). Again, concerning earthworms from which the head had been cut off and several millimeters of the ventral cord removed he says (p. 708): "The head regenerates rather later in these operated animals than in control animals." In the regeneration of the arms of the starfish (p. 711) a similar observation is recorded.

Goldfarb, however, is of the opinion that "any severe injury * * *, whether involving the nerves or any other tissue, retards regeneration." Stockard, on the other hand, concluded that in *Cassiopea*, as Morgan had already shown for a considerable number of animals, the rate of regeneration increased in proportion to the extent of injury and that the deeper the cut—*i.e.*, the nearer to the center of the disk—the faster would be the following regeneration.

In my experiments the amount of tissue removed from the margin of each half of any disk was the same. The differences in result observed were, therefore, due to the difference in kind, not in quantity of tissue removed. As recorded previously, the difference in rate of regeneration is in *Cassiopea* greatest in the early stages and gradually declines throughout its course, at least through the periods followed in these experiments. Goldfarb's observations appear also to show a

similar course of events in earthworms, starfish, and amphibians. This result, on the other hand, is opposed to the conclusion of Child (1910) that "as most experiments not only on the Turbellaria, but on other forms, indicate, it is probable that the early stages of the formation of new tissue are largely or wholly independent of the nervous system." A later part of the same statement is in perfect accord with my result, as follows: "But it is difficult to understand how the nervous system of an adult animal could fail to affect the amount and rapidity of growth in a regenerating part composed largely of muscles and sense-organs. Absence of such an effect would be in direct opposition to the well-established fact of the functional influence of the nervous system on various parts of the organism."

Morgulis (1912) concludes from his experiments on brittle-stars that the presence at the cut surface of the radial nerve, either with or without its being in continuity with the remainder of the nervous system, is a "conditio sine qua non" for normal regeneration and that the presence—purely as a mechanical matter, not the functional activity—of the nerve is the important factor in regeneration. This is in direct opposition to my results. A study of the figures illustrating his paper shows, nevertheless, that the arms in which the nervous connection is undisturbed (control) regenerates most rapidly.

As to the nature of this influence, it is evident from the study of the rate of general metabolism of half-disks with and without sense-organs that it is closely associated with if not identical with the control of the general metabolism of the animal. The latter experiments have not been carried far enough to give a definite answer to the question of whether or not there is a gradual decline in the difference in the rate of metabolism corresponding to that shown in regeneration.

This result clearly supports the general contention of Child, that the influence of the nervous system on regeneration is indirect rather than direct, but does not confirm his statement that there is a direct relationship between the rapidity of regeneration and the "characteristic motor activity of the parts concerned." It is shown, on the contrary, that motor activity may be greatly increased without altering to a proportional extent either the rate of regeneration or general metabolism.

The rate of regeneration appears to be simply one expression of the general metabolic activity of an organism and consequently to be subject to the control of the nerve-centers in the same manner as the many other functional activities—for some of which, at least, a direct nervous control can not be denied.

INFLUENCE OF THE SENSE-ORGANS ON THE LOSS IN WEIGHT DURING STARVATION.

The influence of the sense-organs on the rate of regeneration is so clearly marked when muscular activity is excluded as the determining factor that it seemed apparent that other metabolic activities might be used as a measure of this influence.

Mayer (1914) has shown that when *Cassiopea* is starved in sea-water from which all food material has been removed by either careful filtration or by heating to 71° C. and restoring by distilled water the amount lost by evaporation, the loss of weight follows a course which can be expressed mathematically by the formula $y=w(1-a)x$, in

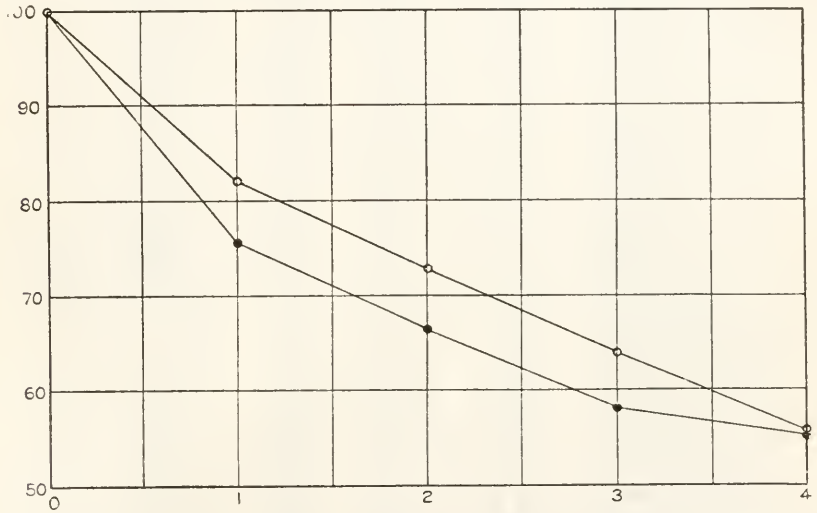

FIG. 10.—Showing loss in weight of active and inactive half-disks during starvation. (Compare table 9.) The divisions along the ordinate represent percentages of the original weight; those along the abscissa represent time in days.

which $y=$the weight on any given day, w the original weight, x the number of days' starvation, and a constant, "the coefficient of negative metabolism." The greater part of the loss in weight appears to be borne by the mesoglœa, since this tissue constitutes by far the greater bulk of the body, as is shown by histological examination of the tissues and chemical analysis of medusæ before and after starvation. The simple character of the law which governs the loss of weight in *Cassiopea* during starvation also indicates that substances of the same sort serve to maintain the medusa throughout nearly the entire course of starvation. As the result of starvation the cells become reduced in size, some degenerate and disappear, while at the same time the mesoglœa becomes highly vacuolated. Within the short period of time covered by these experiments very little histological change could be recognized in the cells. The change in the appearance

of the mesoglœa was, however, much more marked in that vacuolization had become more easily noticeable.

There is clearly a different tissue chiefly involved in the loss of weight during starvation and in regeneration (see page 131), so that

TABLE 6.—*Record for 0 to 4 days.*

[Twenty disks to show the differences in loss of weight during starvation between active and inactive half disks.]

Specimen.	0 day.	1 day.	2 days.	3 days.	4 days.
No. 1 Active	11	7.75	6.25	5.30	4.75
No. 1 Inactive	9.5	7	6.00	4.75	4.50
No. 2 Active	26	20	16.75	14	13
No. 2 Inactive	22	11	15	12.75	11
No. 3 Active	25	19	17	14.75	13.5
No. 3 Inactive	30	24.5	21.5	18	16.25
No. 4 Active	28	23	16.75	17.50	15.5
No. 4 Inactive	30	26	22	19.25	17.75
No. 5 Active	23	18	15	13.5	13.50
No. 5 Inactive	24	20.5	15.5	14.25	12.75
No. 6 Active	21.5	16.5	13.5	12.5	10.25
No. 6 Inactive	19.5	15.5	13.25	11.5	10.25
No. 7 Active	23.5	18.5	16	14	13
No. 7 Inactive	25	20.5	16.75	14.25	13
No. 8 Active	15	11.5	10	8.00	7.25
No. 8 Inactive	16.5	13.5	10.75	9.00	8.25
No. 9 Active	23	18	15	12.75	11.75
No. 9 Inactive	25	20.75	17.5	15	13.00
No. 10 Active	25	19.75	17	15	13.5
No. 10 Inactive	27	22	18.5	16.5	14
No. 11 Active	23	19.25	16	14	12.50
No. 11 Inactive	21	17.25	14.5	12.25	11.50
No. 12 Active	22	18	15	13.5	11.75
No. 12 Inactive	18	14.5	13.25	10.75	9.75
No. 13 Active	29.75	23.25	20.5	17.50	16.00
No. 13 Inactive	27	22.50	18.75	15.75	14.00
No. 14 Active	23	18	15.25	13.1	11.75
No. 14 Inactive	22	18.5	16.00	13.75	12.00
No. 15 Active	28	20.75	17.5	15	13.5
No. 15 Inactive	28	22.75	20	16.25	14.75
No. 16 Active	16	11.5	9.60	8.00	7.00
No. 16 Inactive	15	11.50	9.00	8.00	6.75
No. 17 Active	14	10	8.50	7.25	6.70
No. 17 Inactive	5	12.25	10.25	9.00	7.75
No. 18 Active	23.5	19.25	16.50	15	13.00
No. 18 Inactive	23	18.75	16	13.75	12.75
No. 19 Active	21	16	13.75	12.00	10.50
No. 19 Inactive	20.5	16.75	13	11.50	10.25
No. 20 Active	31	24.5	21.5	19.20	17.25
No. 20 Inactive	31	25.75	22.75	20.00	17.75

these experiments present a standard of measurement of metabolic activity sharply distinguished from that employed in the study of regeneration.

Mayer (*op. cit.*) has pointed out that the value of *a* (the coefficient of negative metabolism) has a different value for practically every set of experimental conditions. Thus this value differs when employing entire medusæ, regenerating portions of oral arms or the bell-margin, disks

from which arms and stomach had been removed, or half-disks. Besides, on account of the relative activity of the zooxanthellæ (symbiotic algæ), the rate of starvation differed greatly when the experiments were carried on in the diffuse light of the laboratory or in darkness.

TABLE 7.—*Record for 0 to 4 days.*

[Twenty disks to show the differences in loss of weight between active and activated half-disks.]

Specimen.		0 day.	1 day.	2 days.	3 days.	4 days.
No. 1	Active	28	20.5	18.00	16.25	14.50
	Activated	29	21	18.50	17.75	16.75
No. 2	Active	29.5	20.5	18.75	15.75	13.00
	Activated	29.25	21.5	20.25	17.50	15.25
No. 3	Active	31	22.5	21.00	19.25	17.50
	Activated	29	21.75	21.00	18.00	16.25
No. 4	Active	31.5	23.00	21.75	18	15.50
	Activated	29.5	23.00	21.75	18.5	16.25
No. 5	Active	29.5	21.00	18.75	18.00	17.25
	Activated	27.75	23.5	20.25	20.00	19.00
No. 6	Active	31.25	23.25	20.00	17.00	15.00
	Activated	31	24	22.50	18.50	15.25
No. 7	Active	20	20	18.75	16.50	14.75
	Activated	32.5	24.5	22.25	20.25	18.75
No. 8	Active	34.5	25.5	23.5	21.25	19.75
	Activated	34	27.5	24.5	22.50	20.75
No. 9	Active	34.5	18.00	16.25	14.50	13.00
	Activated	25.5	18.75	17.50	16.00	15.25
No. 10	Active	24	18.5	16.5	15.25	14.50
	Activated	24.25	19.25	18.00	16.00	14.75
No. 11	Active	24.25	19.25	18.00	15.50	13.25
	Activated	26.25	21.75	21.00	19.25	17.50
No. 12	Active	22.00	17.00	15.25	13.50	12.50
	Activated	22.25	16.50	15.50	14.50	13.75
No. 13	Active	28.5	23.25	21.50	19.25	17.75
	Activated	32.25	24.5	22.80	21.5	20.25
No. 14	Active	32.25	25.00	22.00	20.5	18.75
	Activated	20	24.00	21.75	20.25	19.00
No. 15	Active	34.25	27.25	25.00	22.75	20.25
	Activated	34	27.50	24.25	23.00	21.00
No. 16	Active	37.25	29.25	27.5	24.00	21.75
	Activated	35.75	27.50	24.50	23.25	21.25
No. 17	Active	21.00	15.50	13.50	12.50	10.75
	Activated	26.00	19.75	17.50	16.00	14.75
No. 18	Active	37.00	25.25	23.6	21.50	19.75
	Activated	31.00	25.50	24.5	21.50	20.00
No. 19	Active	21.24	17.50	15.75	13.50	11.25
	Activated	24.00	20.5	18.00	16.00	14.50
No. 20	Active	24.00	16	14.25	13.00	12.00
	Activated	24.00	16.25	16	16.00	15.50

In these experiments, where the disks were prepared by one of the three types of operations previously described, there was no necessity for taking precautions to remove the normal food materials from the water. Water from the regular laboratory supply pumped through vulcanite pipes by a vulcanite pump was regularly used, as no difference was found when water brought in glass jars from the ocean was used.

Mayer has called attention to the fact that regenerating specimens, during the first days of any experiment, starve more rapidly than normal medusæ.

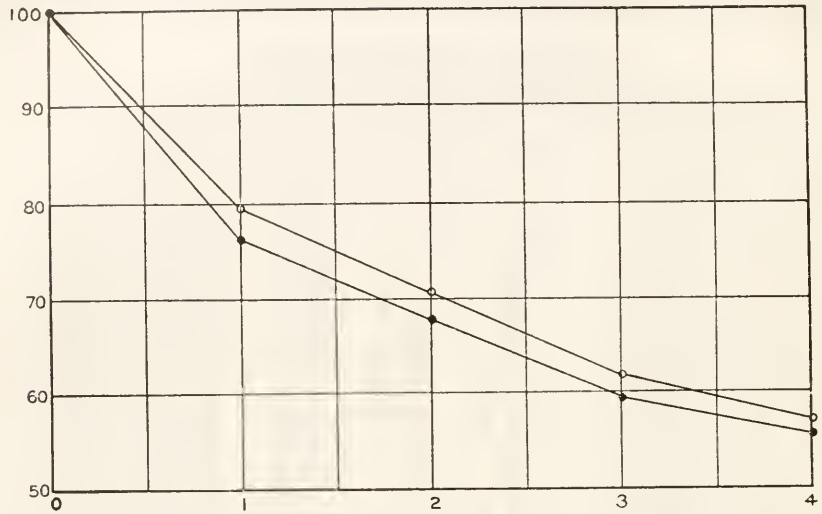

Fig. 11.—Showing loss of weight of active and activated half-disks during starvation. (Compare table 10.) The divisions along the ordinate represent percentages of the original weight; those along the abscissa represent time in days.

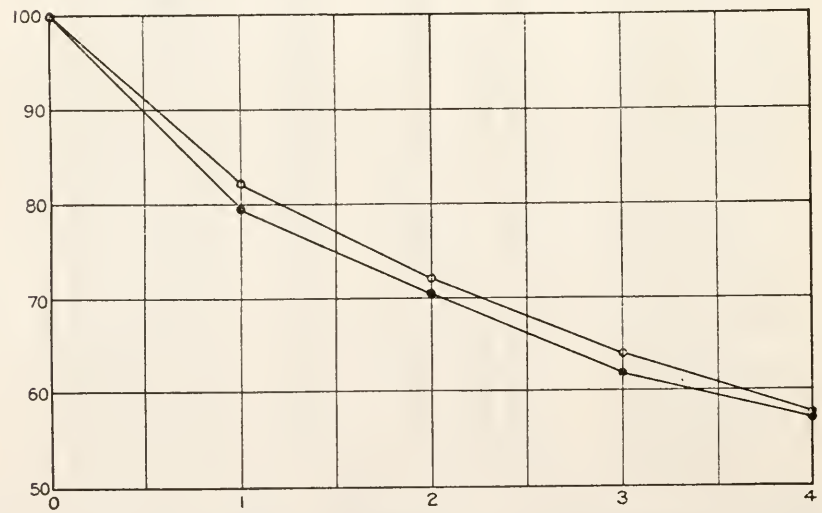

Fig. 12.—Showing loss in weight of activated and inactive half-disks during starvation. (Compare table 11.) The divisions along the ordinate represent percentages of the original weight; those along the abscissa represent time in days.

In the same manner during the first half day of any experiment the member of any pair of half disks which showed the highest metabolic activity showed also the greatest loss in weight, yet even in the course of these experiments the difference in rate became less marked toward

the end of the period of observation. This appears most clearly in experiments in which active and inactive specimens are compared (fig. 10), while the nearest approach to actual convergence is shown in the series in which activated and inactive specimens are compared (fig. 1).

TABLE 8.—*Record for 0 to 4 days.*

[Twenty disks to show the differences in loss of weight between activated and inactive half-disks.]

Specimen.		0 day.	1 day.	2 days.	3 days.	4 days.
No. 1	Activated.....	30.00	24.00	21.75	20.25	19.00
	Inactive.....	29.5	26.25	22.50	20.00	18.25
No. 2	Activated.....	22.25	16.50	15.50	16.50	13.75
	Inactive.....	22.75	19.00	16.25	14.00	12.50
No. 3	Activated.....	26.00	20	17.50	16.25	14.25
	Inactive.....	26.50	22.25	18.50	15.25	13.75
No. 4	Activated.....	28	23.75	20.50	18.75	17.00
	Inactive.....	29.5	25.75	21.75	19.00	16.75
No. 5	Activated.....	17.25	13.50	11.75	10.00	8.75
	Inactive.....	17.75	14.25	12.75	10.25	9.00
No. 6	Activated.....	33.5	27.25	24.50	22.00	20.00
	Inactive.....	35.00	29.00	25.50	22.25	19.50
No. 7	Activated.....	21.5	16.25	15.00	13.75	12.25
	Inactive.....	23.00	18.75	16.25	14.00	12.75
No. 8	Activated.....	23.5	16.00	15.25	14.50	13.00
	Inactive.....	24.00	20.75	16.25	14.50	12.25
No. 9	Activated.....	27.75	23.25	20.00	17.25	13.75
	Inactive.....	29.00	23.50	20.75	17.25	14.75
No. 10	Activated.....	31.00	24.75	23.25	20.75	17.75
	Inactive.....	31.50	26.75	22.75	20.50	17.25
No. 11	Activated.....	24.75	19.25	17.50	15.75	13.00
	Inactive.....	23.50	20.00	17.25	14.75	12.50
No. 12	Activated.....	21.50	16.75	15.00	13.25	11.75
	Inactive.....	22.00	17.75	15.00	13.25	11.50
No. 13	Activated.....	27.00	22.50	19.75	18.00	15.50
	Inactive.....	27.50	22.25	19.75	17.00	14.75
No. 14	Activated.....	23.75	18.00	16.50	14.25	12.00
	Inactive.....	25.00	20.25	17.50	15.25	12.75
No. 15	Activated.....	19.75	15.75	12.50	10.75	9.00
	Inactive.....	20.50	17.00	13.75	11.50	10.00
No. 16	Activated.....	26.25	20.25	17.75	15.25	13.00
	Inactive.....	27.00	22.75	19.00	15.75	13.50
No. 17	Activated.....	20.75	16.50	14.25	13.00	11.50
	Inactive.....	21.50	19.25	16.50	14.75	12.25
No. 18	Activated.....	18.50	15.00	13.25	12.50	10.00
	Inactive.....	20.00	16.00	13.75	11.75	10.25
No. 19	Activated.....	24.50	18.75	16.25	14.00	11.75
	Inactive.....	25.25	21.00	17.75	15.25	13.75
No. 20	Activated.....	27.00	21.25	19.00	16.25	13.50
	Inactive.....	28.00	23.00	19.75	16.25	13.75

The previous researches on inanition in invertebrates have dealt with either the chemical or histological changes brought about by the disturbances in normal metabolism caused by this condition, and need not be reviewed in this connection. In general, it has been observed that there is a shrinkage in size and frequent degeneration of the cells of all parts of the body. The epithelial tissues in general have a tend-

ency to lose their cell-wall and to become a syncytium. Usually the loss of material is not nearly so selective as in the vertebrates; practically all organs become involved in the loss in an early stage of inanition, except in forms in which there is some special storage tissue, as the fat-bodies of insects.

The much more extensive literature dealing with inanition in vertebrates shows that the loss of body-substance takes place in a highly selective manner, involving many tissues before any marked loss is undergone by the "vital organs." Several researches in this field have demonstrated the importance of metabolic muscular activity as a factor in determining the rate of tissue destruction. The usual course of the changes involved in inanition is not, however, greatly altered by differences in the rate of metabolism, the one factor most influenced being the rate of occurrence of these changes.

TABLE 9.

Days after operation.	Loss of weight in active and inactive half-disks.		Loss of weight in active and activated half-disks.		Loss of weight of activated and inactive half-disks	
	Weight of half with sense-organs.	Weight of half without sense-organs.	Weight of half with sense-organs.	Weight of activated half.	Weight of activated half.	Weight of half without sense-organs.
0 day.....	100	100	100	100	100	100
1 day.....	75.34	81.81	76.29	79.41	79.47	81.97
2 days....	66.72	71.27	67.18	70.58	70.83	71.84
3 days....	58.54	64.09	59.76	61.99	62.03	63.91
4 days....	55.27	55.41	55.88	57.18	57.21	57.23

The results of three series of experiments, in each of which the halves of 20 disks were prepared in such a manner that they fell into one of the three categories described on pages 127 and 128, are presented in tables 6, 7, and 8, and figures 10, 11, and 12.

A summary of the results for each of the series of experiments is given in their proper order in table 9, in which the weights are reduced to terms of 100 grams for the original weight of each set of half-disks in each series. A comparison of these tables and the curves for the loss of weight with those given for the rates of regeneration under similar operations show a striking similarity in the results when those obtained by the use of these standards of measurement are compared, although, as previously stated, the tissues chiefly concerned in the two forms of metabolic activity are fundamentally different.

Table 10 gives the record of the halves of 10 disks prepared to give active and inactive specimens and shows loss in weight, decrease in diameter, measured for each half-disk at the line of separation of the halves, and shrinkage in the diameter of a cavity originally 22 mm. In several instances the diameters of the halves of the same disk vary 1 to 3 mm. on account of irregularities in the outline due to the cuts

made in removing the sense-organs or the similar-shaped pieces of tissue between the sense-organs of the active half-disk.

The results of the measurements of size are completely in accord with those obtained in studying the loss in weight of the same half-disks, or the larger series of specimens prepared in the same manner, as shown in table 6. Although in the greater number of regeneration experiments no careful measurements were made of the decrease in the diameter

TABLE 10.—*Record for 0 to 4 days.*

[Ten disks separated into active and activated halves, to show the comparative loss in weight, decrease in diameter, and decrease in diameter of the cavity where a disk of tissue had been removed in order to measure the amount of regeneration.]

Specimen.	0 day.			1 day.			2 days.			3 days.		
	Weight.	Diameter of disk.	Diameter of cavity.	Weight.	Diameter of disk.	Diameter of cavity.	Weight.	Diameter of disk.	Diameter of cavity.	Weight.	Diameter of disk.	Diameter of cavity.
No. 1 Active....	37	132	22	29	108	12	26	118	11	24.5	106	10.5
No. 1 Activated.	38			32	118	12	29	114	12	27	111	11.75
No. 2 Active....	18	110	22	13	83	11	11	75	9	10	68	8.25
No. 2 Activated.	21			16.5	99	14	14	89	11.5	12	80	11.00
No. 3 Active....	37	137	22	26	110	12	23	100	10	20.5	92	9.00
No. 3 Activated.	38			32	127	17	27	113	11	23.5	103	9.50
No. 4 Active....	31	130	22	22.5	98	13	21.5	93	9	18	87	8.00
No. 4 Activated.	31			26.5	118	17	23	110	11	20.5	104	9.75
No. 5 Active....	24	118	22	18	96	11	16	86	10	14.00	78	8.50
No. 5 Activated.	24			18	110	19	16	94	12	14.00	85	10.75
No. 6 Active....	20	110	22	14	86	13	12	79	10	10.00	71	8.75
No. 6 Activated.	20			15.5	100	16	14.25	92	13	11.5	81	11.50
No. 7 Active....	28	125	22	21.5	109	13	19	98	10	16	89	8.00
No. 7 Activated.	27.5			23	117	16	20	105	12	18	99	9.50
No. 8 Active....	28	123	22	21	93	12	19	88	10	16	84	8.25
No. 8 Activated.	30			25	118	17	21.5	112.5	12	19	107	10.50
No. 9 Active....	28	122	22	22.5	110	11	19.5	95	11	16	86	10.50
No. 9 Activated.	25			20	110	15	17	97	12	14.5	90	10.75
No. 10 Active....	28	125	22	22	112	13	19	99	11	16.5	91	9.75
No. 10 Activated.	29			26	120	18	23	107	13	19.5	98	11.25

of the cavity in which the amount of regeneration was measured, the differences in this regard between the different types of specimens was easily distinguishable in all cases and was always parallel to the relative rate of regeneration shown by either one of a given pair of half-disks, by whichever of the three operations they had been prepared.

INFLUENCE OF SENSE-ORGANS ON THE RATE OF GENERAL METABOLISM AS MEASURED BY CO_2 PRODUCTION.

The results of the experiments recorded in the two preceding sections show that the nerve-centers exert a decided influence on the rate of metabolic activities when measured by two different standards. It seems apparent also that, although the standards of measurement adopted—regeneration and loss of weight during starvation—largely

involve the activities of distinct tissues, the influence of the sense-organs is exerted in a manner strikingly similar in the two cases. This similarity in result suggests the probability that the effects measured are only different phases of the general metabolic activity of the organism, which when measured as a whole would show an influence of the nerve-centers comparable to that obtained when the activities involved in distinct functions are used as the unit of measurement.

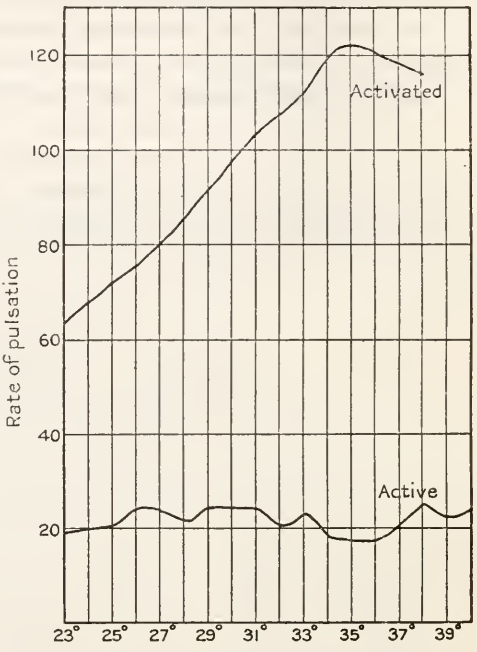

FIG. 13.—Showing effect on rate of pulsation of active and activated halves of the same medusa disk when subjected to an increase in temperature from 23° to 40° C. The upper curve is for the activated specimen; the lower curve for the active specimen.

The measurement of the amount of carbon dioxide given off by a half-disk within a given space of time affords means of determining the comparative rate of total metabolic activity of specimens under the operative conditions used in the previous experiments. The technique of the experiments was as follows: The half-disks were prepared in the manner previously described, so that active, activated, and inactive individuals were secured for comparison. Each half-disk of any pair was placed in a separate jar containing about 1,200 c.c. of fresh sea-water brought from the ocean at the time of beginning a set of experiments. The capacity of the jars varied slightly and pairs of equal volume had to be selected for the two halves of the same disk.

As the measurable amount of CO_2 produced and consequently the rate of pulsation for some time after the beginning of an experiment were largely dependent upon the activity of the zooxanthellæ, most of the experiments were started late in the afternoon and therefore continued during the hours of darkness. When experiments were going on during the day the necessity for covering the jars with dark cloth caused a decided rise in temperature, which increased unnecessarily the complication of the calculations involved in determining the amount of CO_2 given off in respiration.

Preliminary experiments along this line were undertaken, in collaboration with Dr. S. Tashiro, in 1915, when the determinations were

made by means of titrating the sea-water with n/100 NaOH, using phenolphthalein as an indicator and titrating to the color obtained by adding the same amount of indicator to an equal volume of sea-water taken as a control at the beginning of each experiment.

In the later experiments the amount of CO_2 given off by each half-disk was determined by ascertaining the P_H (minus log hydrogen-ion concentration) of a sample of sea-water taken as a control at the beginning of the experiment, and that from the jar in which the specimen had been respiring during the experiment. Both specimens involved in an experiment were removed from the jars at the same time, whenever the determinations were to be made or either specimen had ceased pulsating. The volume of CO_2 necessary to bring about a corresponding change in the hydrogen-ion concentration of an equal volume of sea-water (1,200 c.c.) was determined by adding known volumes of CO_2 to that volume of sea-water.

The results of these determinations are shown in figure 13. This result confirms McClendon, who shows that the CO_2 content of sea-water can be more accurately measured by determining the hydrogen-ion concentration than by a direct determination of the CO_2.

When the half-disks were placed in the closed jars the increase in the hydrogen-ion concentration of the sea-water caused at first an increase in their rate of pulsation. This was followed after 3 or 4 hours by a gradual decline in rate until finally both activated and active half-disks had been completely narcotized by the CO_2 which they had themselves given off. The decline of the rate of activated half-disks was very regular and the pulsations became gradually weaker, until scarcely discernible before entirely ceasing. The active specimens, on the contrary, behaved very erratically. Frequently when the pulsation had become very slow, or had even ceased for several minutes, these specimens would suddenly take up rapid pulsation and for a short time their activity would be even greater than when under the influence of the first stimulating effect of the increased hydrogen-ion concentration in the early part of the experiment.

The activated half-disk was invariably the first to become narcotized, the muscles in these, as in all other experiments involving anesthetics, being less resistant to such agents than the sense-organs.

The activated specimens, when once their pulsations had ceased, were incapable of again taking up pulsation unless stimulated to activity by artificial means. The active specimens, even after having been quiescent for several hours, would take up pulsation within 2 minutes after being put into fresh sea-water.

When such experiments were carried on in daylight narcosis would not be produced during the first day, because of the reduction of the amount of CO_2 in the water by the photosynthetic activity of the zooxanthellæ.

The disks were quickly narcotized on being put into sea-water to which had been added enough CO_2 to change the hydrogen-ion concentration to that shown by the water in the jars in which the half-disks had ceased pulsating. It seems apparent, therefore, that narcosis had been caused entirely by the increase in CO_2 and not by accumulation of other waste products of metabolism, or by a decrease in the amount of oxygen available for respiration.

Table 11 shows the results of a typical experiment for determining the total metabolism of active and activated halves of the same disk. The hydrogen-ion concentration is given in terms of the P_H numbers of Sorensen. The sea-water at the beginning of the experiment had a hydrogen-ion concentration of $P_H=8.1$ ($C_H=0.793\times10^{-8}$).

TABLE 11.

Specimen.	Weight.	Pulsation-rate.			P_H.
		$1^h\,45^m$ p. m.	$4^h\,40^m$ p. m.	$7^h\,30^m$ p. m.	
	grams.				
No. 1, *a*.....	22.8	44	62	18	7.80
No. 1, *b*.....	23.0	128	136	120	7.90
No. 2, *a*.....	30.0	36	34	22	8.00
No. 2, *b*.....	30.5	88	86	98	8.00
No. 3, *a*.....	39.0	44	36	8	7.90
No. 3, *b*.....	41	130	158	126	7.90
No. 4, *a*.....	28	56	22	25	7.90
No. 4, *b*.....	29	96	106	Out.	8.00
No. 5, *a*.....	29.25	84	32	32	7.90
No. 5, *b*.....	28.75	116	120	132	7.90

These experiments were not carried out in sufficient detail to determine the regular course of respiration or the normal respiratory quotient under the influence of varying hydrogen-ion concentration, their aim being to determine the influence of the sense-organs on the rate of total metabolism.

The differences due to muscular activity conform to those obtained from the measurements of rate of regeneration and loss of weight during starvation. The amount of respiration of an active half-disk was invariably as great as its activated mate in spite of the great differences in muscular activity. Indeed, in only a very few instances did the normal active specimen show as low a CO_2 production as the more rapidly pulsating activated half of the same disk.

The activated half-disks were narcotized when the hydrogen-ion concentration of the sea-water in which they were contained had reached about 0.126×10^{-7} ($P_H=7.9$). In fact, a greater concentration of hydrogen ions was seldom reached in the water in which the activated disks were kept, as after the animals had been sufficiently narcotized to cause the cessation of pulsation other metabolic activities

were also interfered with to such an extent that respiration practically ceased. Active half-disks were capable of maintaining at least intermittent pulsation until the hydrogen-ion concentration had become 0.159×10^{-7} ($P_H=7.8$). Nearly all the determinations of the H concentration were made by means of color comparison with tubes of known H concentration prepared by Hynson Westcott & Dunning, from buffer mixtures standardized by J. F. McClendon, containing mixtures of phosphates and borates using thymolsulfonephthalein for concentrations above $P_H=8$ and phenolsulfonephthalein for concentrations below $P_H=8$. These tubes were in no part of their range graduated more closely than $P_H=0.1$, so that no very fine readings could be obtained except by estimating fractions of this unit, which could not be done with any considerable degree of accuracy.

INFLUENCE OF SENSE-ORGANS ON THE CHANGE IN RATE OF PULSATION IN RESPONSE TO CHANGES IN TEMPERATURE.

The studies of Harvey (1911) and Mayer (1913) have shown that when *Cassiopea* is slowly heated the rate of nerve-conduction increases from about 18° C. to about 36° C., after which there is a rapid decline in the rate. Recovery of the medusa is impossible if the temperature is carried much above 40° C.; indeed, when cooling is begun before a fatal temperature is reached the normal rate of pulsation does not return even when the medusa has been kept at normal temperature for several days. In its general features the curve for the rate of nerve-conduction in *Cassiopea* resembles that for enzyme action, as was pointed out by Harvey, and as shown for the pulsation-rate of the vertebrate heart (Knowlton and Starling (1912) *et al.*).

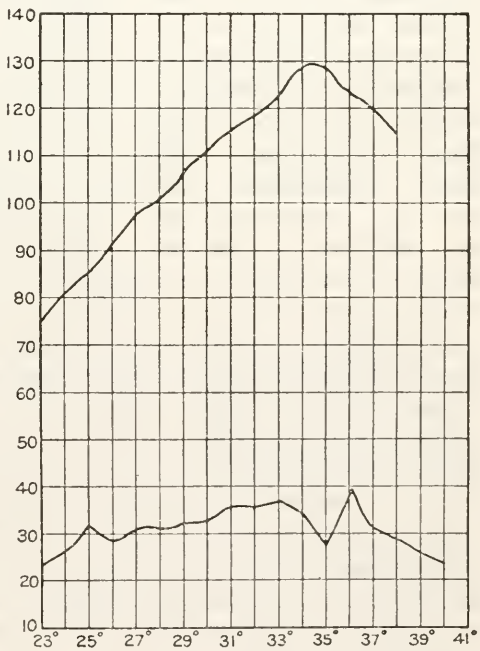

FIG. 14.—Curves showing averages of all determinations of effect of change in temperature on active and activated halves of the same disk.

Since Mayer (1908) has shown that the initiation of the stimulus for pulsation takes place in the sense-organs through a definite chemical reaction, it was thought possible that if a sense-organ were subjected to changes in temperature while the body of the disk was kept at a

constant temperature, the resulting rate of pulsation might follow Van't Hoff's law more closely than when the entire disks were subjected to the change in temperature, or the rate of pulsation—nerve-conduction—of activated disks under similar conditions was measured. Such experiments showed, however, that the results from this cause were in no essential different when either a single sense-organ, an "active" disk, or activated disk were subjected to the changes in temperature. All my later experiments were therefore confined to those in which active and activated halves of the same medusa disk were subjected to the temperature changes at the same time in order to determine the influence of sense-organs upon the response to changes in temperature. The prepared half-disks were placed in a 4-liter jar of fresh sea-water which was in turn contained in a 20-liter jar of fresh water supported on a copper tray filled with water. The water surrounding the inner jar was cooled by the addition of ice or heated by the flame of a small alcohol lamp so that a change of 1° in the temperature of the sea-water was obtained in 15 minutes. The activity of the medusæ was sufficient to agitate the water to such an extent that at no time could a difference of 0.1° C. be detected in the temperature of any two portions of the contents of the jar.

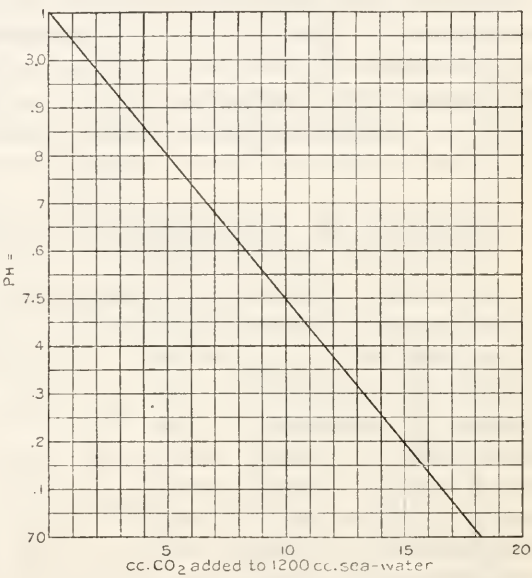

FIG. 15.—Showing change in hydrogen-ion concentration when a known volume of carbon dioxide is added to 1,200 c.c. of Tortugas sea-water.

In beginning any experiment the temperature was lowered to the desired starting-point and then raised until either the desired upper limit was reached or continued until the disks became inactive. On being cooled below 20° C. the activated specimens often ceased pulsating and could not again be aroused to steady pulsation by the application of repeated stimuli until the temperature had been raised to about 22° C. From 23° to 33° C. the increase in rate of pulsation for activated specimens followed a right line (fig. 14) and within that limit the rate was nearly doubled. The active half-disks, over the same range of temperature, gave generally a smaller increase in rate and the change in rate was always more erratic than that shown by those deprived of their sense-organs. (Fig. 14.)

The pulsation-rate of active specimens frequently reached its maximum at from 27° to 29° C. In other specimens the maximum rate was not attained until a temperature of 37° C. had been reached, after which the decline in rate was precipitous, death occurring at 38.5°.

A summary of all the results is given in figure 15, which shows the average for 11 active and 9 activated half-disks.

RELATION BETWEEN AREA OF TISSUE ENERVATED BY A SINGLE SENSE-ORGAN AND RATE OF PULSATION.

Eimer (1874) made the observation that as the area of tissue enervated by a single sense-organ was progressively decreased there was a corresponding decline in the rate of pulsation. He states that the decline in rate is directly proportional to the decrease in area through the range of the experiment.

Romanes (1895) repeated these observations and denied the conclusion of Eimer that the decline in rate was directly proportional to the decline in area; but he did not give any definite statement of the mathematical relation between area and rate of pulsation. Mayer (1906) confirmed and extended these latter observations, but again did not accurately determine the relation between tissue area and rate of pulsation.

In my own experiments all sense-organs but one were removed from each disk, and after allowing a period of 1 hour for recovery from the shock of the operation the rate of pulsation was recorded. Immediately after this record was completed the area under the control of the sense-organ was reduced to one-half its former extent by making a cut through the subumbrella tissues. In this manner the area of tissue was reduced to $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, and $\frac{1}{16}$ of that of the entire disk (fig. 16) and a record of the pulsation-rate was secured for each area. In all, 141 disks were used in this series of experiments.

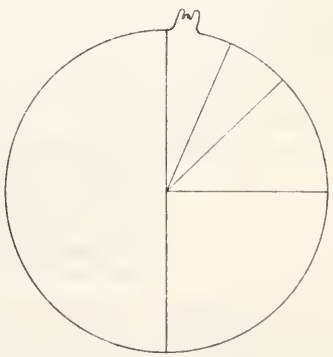

Fig. 16.—Showing method of operation employed in studying the relation between area of tissue innervated by a single sense-organ and the rate of pulsation.

A summary of the results is given in table 14 and figure 17.

As will be seen from the graph, the decline in rate follows a right line very closely, except at the point where the rate for $\frac{1}{8}$ the original area is shown. When extended to included areas of tissue less than $\frac{1}{16}$ of the original the decline in the pulsation-rate follows practically the same formula as shown in figure 17, by the broken line extending to a point where the tissue area under the control of the single sense-organ is reduced to $\frac{1}{64}$ the original size.

A reduction of the area of a disk beyond $\frac{1}{16}$ of the original could not be made accurately by the operation illustrated in figure 17.

When further reduction in area was desired, linear strips of constant width, each with a single sense-organ, were cut from the body of large medusæ, so that the area could be accurately reduced to $\frac{1}{32}$, $\frac{1}{64}$, $\frac{1}{128}$ and $\frac{1}{256}$ that of the original area. The fall in rate, as recorded for the few observations made, when graphically represented produced a continuation of the straight line obtained from the larger subdivisions of the disks.

The elimination of any portion from the excitable area could be produced by immersing that portion in a 0.4 m $MgCl_2$ solution, as shown in figure 18. When the anesthetizing solution was contained

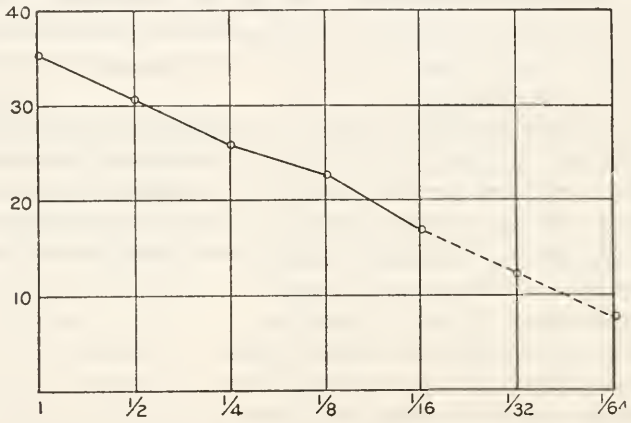

FIG. 17.—Showing graphically the results obtained from the study of 140 disks when the area of tissue was successively reduced to $\frac{1}{16}$ the original area. The broken line extending to an area $\frac{1}{64}$ the original is based on a smaller number of observations. The divisions along the ordinate represent the number of pulsations; those along the abscissa represent the area of tissue under the influence of the sense-organ.

in the middle vessel the nerves were capable of transmitting the impulse across this area, where the muscles were inactive, to the tissue in dish No. 3, so that the inactive area could be obtained at any point in the strip of tissue. No differences in the results were obtained when the activity of a given area of muscles was eliminated by the cutting of the subumbrella tissues or their immersion in the $MgCl_2$ solution. Pieces of tissue in sea-water were found to be capable of responding to each separate induction shock when applied at the rate of 126 per minute when they had been reduced to $\frac{1}{512}$ of the original area of the strip which represented scarcely more than $\frac{1}{10000}$ ($\frac{1}{9817.48}$) of the area of the original disk.

This fact makes it apparent that the decline in rate of pulsation caused by reducing the area enervated by a single sense-organ is not at all dependent upon the necessity for a latent period on the part of the muscular tissues. On the other hand, there is no apparent reason why the sense-organs should be incapable of carrying on at the usual rate the

chemical reaction by which sodium is liberated, unless they are dependent upon the other tissues of the subumbrella for some substance which in the smaller areas becomes reduced to such an extent that it can no longer be produced rapidly enough to allow the sense-organ to discharge at the usual rate.

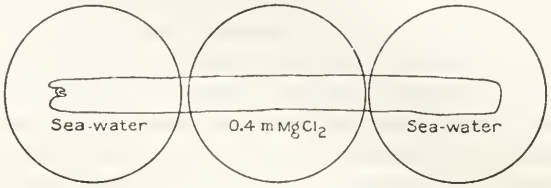

Fig. 18.—Showing arrangement of vessels by which any area of tissue could be rendered inactive in order to determine whether or not the method by which the area of muscle under the influence of a single sense-organ, was reduced was an important factor in determining rate of pulsation.

The fact that in a normal medusa the initiation of pulsation is taken up by one after another of the sense-organs at irregular intervals may indicate that, even while in normal relationship to the surrounding tissues, each sense-organ during its period of control of the pulsation of the medusa exhausts from its immediate surroundings some substance necessary for the maintenance of the discharge of impulses at a regular high rate. Since the pulsation-rate of a medusa is under normal conditions constant, each sense-organ must give up its control as soon as its power of giving out impulses falls below that necessary to maintain the usual rate of pulsation. When a given sense-organ has surrendered to a more active neighbor the initiation of pulsation, it will have a sufficient latent period to recover the chemical equilibrium necessary for bringing its rate of discharge of impulses up to that necessary to supplant any other center as the controlling factor in pulsation.

GENERAL DISCUSSION.

THE RELATION OF MUSCULAR ACTIVITY TO THE RATE OF METABOLIC PROCESSES IN CASSIOPEA.

(1) The relation of muscular activity to the rate of regeneration has been considered in the section dealing with the regeneration experiments and will be here taken up only in making comparison with the results obtained by other methods. It appears to be clearly established that in this organism muscular activity plays a relatively unimportant part in controlling the rate of metabolism as measured by the total CO_2 output or by either of the two other standards of measurement employed. The relatively small difference between activated and inactive specimens as measured by either of the three standards may be accounted for on the ground of differences in temperature induced by the extreme activity of the muscular system. While no estimate of the amount of heat generated through muscular activity is available for *Cassiopea*, the evidence secured from the study of the change in rate of nerve-conduction (pulsation) indicates that a relatively small temperature increase would be sufficient to account for the observed differences. In spite of this temperature effect, activated disks, pulsating at from 3 to 10 times the rate of active specimens, show metabolic activities (by whichever of our three standards measured) considerably lower than do the other halves of the same original disk, the activities of which are under the control of the sense-organs.

(2) With the elimination of muscular activity as an important determining factor in metabolic activity there remains to be considered the nature of the nervous control over metabolic activity. After 1851 (when Ludwig proved the existence of nerves producing activity of the cells of secreting glands) it was held for some time that there were nerve-fibers presiding over other metabolic activities, as growth and repair. With the increasing knowledge of the part played by chemical excitation in controlling many activities, the conception of the trophic function of nerves, in the broader sense, has been discarded by most modern students of physiology.

The trophic function in the special sense of Heidenhain (1858) has been clearly demonstrated by the researches of Babkin (1913) for the nerves of cranial origin to certain gland-cells.

Most studies on the trophic effect of nerves on growth or other general metabolic phenomena have given negative results. Jacobson (1910) concluded, from experiments on pigeons and dogs, that the denervated areas healed as rapidly as those with the normal nerve-supply. Tschermak (1910), after extended researches and a critical review of the literature on trophic and tonic innervation, states that there is no evidence for the existence of separate or specific trophic nerves. He considers it probable, however, that motor and sensory

nerve-fibers convey trophic impulses as an accessory function, which would imply the necessity of qualitative differences in the impulses which traverse any fiber. On the other hand, the work of Head and Campbell, on *Herpes zoster*, supports the conception of a trophic function of nerve-fibers. In this disease the presence of change in the dorsal root ganglia causes abnormal impulses to be sent in an efferent direction along the sensory fibers of the skin. On account of this excitation, blistering of the skin takes place over the area of distribution of the fibers (axones) terminating in the affected ganglia.

Jacobson (*loc. cit.*) states that "in the lower animals the maintenance of life processes are largely a phenomenon of chemical coordination," and that trophic influences ought to be greater in the higher than in the lower animal phyla. Her own experiments showed, in her opinion, that there was no difference in this respect between pigeons and dogs, both animals giving negative results.

Among the lower invertebrates, in *Cassiopea* (and in this respect it is unlikely to differ markedly from other scyphomedusæ) the general metabolic activities, including regeneration (a form of growth), total CO_2 production, and the using of body-substances for metabolism when no food is being taken, are strongly influenced by the condition of presence or absence of some sort of impulses from the sense-organs (nerve-centers). That there are neurones which are specific conductors of trophic impulses as distinct from motor or sensory nerves is by no means implied by this statement. In fact, in a nervous system such as that of the medusæ it is not always possible, on morphological grounds alone, to separate those neurones which are sensory from those of a motor function. In the absence of even this means of establishing the identity of the character of a portion of the neurones it is useless to attempt, in the present state of our knowledge of the anatomy of this nervous system, to make any distinction between the character of the function of any particular portion of it.

In spite of this lack of completeness in our knowledge of the nature of the nervous system of *Cassiopea*, it should be emphasized that, through whatever morphological mechanism it may be exercised, the trophic influence of the sense-organs is a constant factor in the normal metabolic activity. Without these impulses, even while muscular activity is far above normal, all the measurable metabolic activities except that of the motor system are maintained at a considerably lower level than when under the control of the sense-organs, by which muscular activity is held at a much lower rate than that at which these tissues are capable of maintaining a constant activity for long periods.

SUMMARY.

(1) The experiments with entire disks, when the rates of regeneration of specimens on which the sense-organs remained are compared with those of specimens from which all sense-organs are removed, are inconclusive because of wide differences in physiological activity between different individuals.

(2) When we compare the insulated halves of a disk, on one of which the sense-organs remain, while all of them have been removed from the other half, it is found that the half-disk with sense-organs always regenerates most rapidly. This is especially noticeable in the early stages of regeneration. The difference in rate falls gradually throughout the course of an experiment (table 1 and fig. 6).

(3) When disks prepared as in the experiments mentioned in the previous paragraph are allowed to regenerate in sea-water plus 15 parts 0.6 m $MgSO_4$, the regeneration is at first more rapid from the half on which the sense-organs come under the influence of the anesthetic, and from that time on the rate of regeneration is practically equal from both halves (table 2 and fig. 7).

(4) When all the sense-organs are removed from a disk and the halves insulated, muscular activity may be maintained in one half by forming an endless labyrinth of the subumbrella tissue and initiating a circuit wave of contraction by induction shocks. Under these conditions the regeneration is faster from the activated than from the inactive half-disk. The difference is, however, not nearly so great as when the sense-organs are removed from only one of the insulated halves of a disk (table 3 and fig. 8).

(5) Comparison of rates of regeneration of the halves of a disk, one half of which retains its sense-organs, while a circuit wave of contraction is maintained in the muscles of the other half, shows that the half-disk the muscles of which are contracting under the control of the sense-organs regenerates faster, although the rate of pulsation of the activated half is more than 3 times that of the former (table 4 and fig. 9).

(6) The influence of the nervous system on the earlier stages of regeneration has been noted by several earlier investigators, but apparently no importance has been attached to it.

(7) These experiments indicate that the rate of regeneration is simply one expression of the general metabolic activity of an animal, and as such is subject to the influence of the nerve-centers, as are many other functional activities.

(8) When a normal medusa, a regenerating medusa, a medusa disk, or half-disk is kept in water free from food material, the loss in weight follows a curve of the formula $y = w(1-a)x$. Here y = the weight for any given day, w = original weight, x = number of days of starvation, and a is a constant, the "coefficient of negative metabolism." The value

of *a* will differ under different experimental conditions, but all observed instances agree closely with the expectations.

(9) The greater part of the loss in weight appears to be shown by the mesoglœa, which undergoes marked histological changes during the course of starvation.

(10) As in regeneration experiments, the greatest metabolic activity is shown by the active specimens, the least by the inactive, while the activated individuals stand in an intermediate position, being closest to the one in which muscular activity is entirely suppressed. (Table 9, and figures 10, 11, and 12.)

(11) Specimens as prepared for the previous experiments were kept for various periods in closed jars of sea-water, each containing about 1,200 c.c. The amount of CO_2 given off was ascertained by determining the hydrogen ion concentration of this water at the beginning and end of each experiment. The volume of CO_2 necessary to bring about the observed change was determined by adding known volumes of this gas to 1,200 c.c. of sea-water, as shown in figure 13.

(12) While the rate of pulsation of the activated half-disks was generally more than 3 times that of the active half of the same disk, the amount of CO_2 given off in respiration from the activated specimen was never more, and almost always considerably less, than that from the active specimens of any pair of half-disks. In general the results from this type of experiment are in perfect accord with those obtained from those recorded in sections I and II, where the standard of measurement was a component part of that used in the respiration experiments.

(13) The change in rate of nerve-conduction (pulsation) in activated specimens over a range in temperature from 23° to 33° C. follows nearly a straight line, the actual rate being approximately doubled for this increase of 10° C. Beyond 35° the rate rapidly declines, death ensuing at 38°. The curve for this change resembles very strikingly that for enzyme action. The change in rate of active half-disks is much more erratic. Such specimens can, however, be carried to much lower temperatures (14° C.) before activity ceases, and the upper limit of temperature was always higher by 2 or 3 degrees than for the activated half of the same disk. When a single sense-organ was subjected to changes in temperature, the remainder of the disk, without its sense-organs, being maintained at a constant temperature, the results showed no characteristic difference from those obtained when the whole disk was subjected to the temperature change.

(14) As the area of tissue enervated by a single sense-organ is successively reduced to one-half its former extent at each operation, the rate of pulsation declines until, when the area has been reduced to $\frac{1}{16}$ its original extent the pulsations will have been reduced to approximately half their original number. Beyond this point the reduction in rate of pulsation followed the same ratio to the area, at least until the final

area of $\frac{1}{128}$ its original extent. A piece of subumbrella tissue 0.0001 the original area of the disk is still capable of responding to each induction shock when applied at the rate of 126 per minute. Apparently, therefore, the necessity for a latent period on the part of the muscles is not an important factor in the decline in rate of pulsation. It appears more probable (as indicated by the manner in which each of the 16 sense-organs, usually present on a medusa, take up in turn the initiation of the stimulus for pulsation) that some chemical interchange between the sense-organs and the surrounding tissue is necessary in order that the activity of these structures shall be maintained at the highest state of efficiency. As the tissue area is reduced this readjustment takes place at a correspondingly lower rate, because the material essential for this adjustment is less readily available to the sense-organs.

(15) The evidence from the foregoing experiments establishes the fact that some sort of trophic influence is exerted in general metabolic activities by the sense-organs. The structure of the nervous system of *Cassiopea* makes it impossible, however, to prove the existence of trophic nerve-fibers as distinct from those of sensory or motor function. On the other hand, it is shown that in this lowly organized form there are transmitted from the nerve-centers certain influences that are absolutely essential for maintaining general metabolic activities at their normal rate.

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VIII.

THE CHEMISTRY OF LIGHT-PRODUCTION IN LUMINOUS ORGANISMS.

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One text-figure.

CONTENTS.

PAGE.

Introduction 173
Studies on the ostracod crustacean *Cypridina hilgendorfii:*
General character of light and of luminous gland 174
Nature of the light-secretion 175
Porcelain filtration 175
Lack of oxygen 176
Desiccation and ether extraction 176
Chemical tests on the secretion 176
Luciferin and luciferase 176
Photophelein and photogenin 178
Distribution of photophelein and photogenin in organisms 179
Dialysis 183
Adsorption 183
Temperature 183
Stability 184
Preservatives and anesthetics 184
Protein precipitants 186
Acids and alkalies 186
Potassium cyanide 188
Saturation with sugar, $(NH_4)_2SO_4$, and NaCl 188
Concentration of photogenin and photophelein in *Cypridina* 188
Is photogenin used up in light-production with photophelein? 189
Summary of results from *Cypridina* 191
Studies on fireflies:
Desiccation and extraction with fat solvents 193
Extraction with aqueous solvents and effect of oxygen 195
Distribution of photogenin and photophelein in the firefly and other organisms 197
Concentration of photogenin and photophelein necessary to produce light 199
Properties of photogenin and photophelein 199
Summarizing table 200

PAGE.

Studies on a pennatulid, *Cavernularia haberi:*
General characters of the animal and of the photogenic material 202
Electrical stimulation 203
Lack of oxygen 204
Reductase, oxidase, and catalase 204
Temperature 205
Photogenin and photophelein 205
Chemical reactions 207
Anesthetics 207
Potassium cyanide 208
Summary of results from *Cavernularia* 208
Studies on luminous bacteria:
Desiccation and extraction with fat solvents 209
Effect of lack of oxygen 212
Photogenin and photophelein 214
Temperature 215
Oxidase and catalase 216
Luminous bacteria and osmotic pressure 216
Luminous bacteria and balanced solutions 217
Effect of acids and alkalies 218
Effect of homologous alcohols (anesthetics) 219
Effect of potassium cyanide 220
Summary of results from luminous bacteria 220
Studies on a squid, *Watasenia scintillans:*
General characteristics of light-production 222
Photogenin and photophelein 223
Studies on a fish, *Monocentris japonica* 223
Studies on oxidative chemiluminescent reactions with substances of definite composition:
Historical 224
The chemiluminescence of pyrogallol 225
Significance for bioluminescence 232
Summary of study of chemiluminescent reactions 232
General conclusions 233
Bibliography 234

THE CHEMISTRY OF LIGHT-PRODUCTION IN LUMINOUS ORGANISMS.[1]

By E. Newton Harvey.

INTRODUCTION.

The literature on the subject of animal phosphorescence or bioluminescence or biophotogenesis is so voluminous that no attempt can be made in the present article to record a complete list. A fairly complete bibliography will be found in Mangold's article, "Die Production von Licht," in Winterstein's *Vergleichende Physiologie*. The names of Beijerinck, Dubois, Ehrenberg, Krukenberg, Mangold, McDermott, Molish, Panceri, Phipson, and Quatrefages are intimately connected with the chemical and physiological side of light-production. The work which has been accomplished up to the present time indicates that light-production by animals is to be classified among the chemiluminescent reactions which have been studied from the purely chemical side, especially by Radziszewski and Trautz. All of the evidence now indicates that oxygen is necessary for this reaction—at least the statement is true for the following investigated forms: the fireflies; luminous bacteria and fungi; *Noctiluca* (2); a pennatulid (*Cavernularia*); and an ostracod crustacean (*Cypridina*). It has been objected by some that if we place the organisms, say luminous bacteria, in an atmosphere devoid of oxygen and find that no light is produced, that merely means that certain functions of the cell are interfered with, *including* light-production, but does not indicate that oxygen is actually used up in the photogenic process. The objection will not hold, however, for extracts of luminous cells or the luminous secretion of *Cypridina*, which contains the light-producing substance in solution (so that it can not be removed by filtration through a porcelain or siliceous filter) will give no light if the oxygen be removed, and light returns when oxygen is readmitted.

We may therefore say that a definite substance is burned (oxidized), and in some forms it burns as granules and in others as a homogeneous and probably colloidal solution. This substance is stable and nonluminous when dried and is not a fat or any substance soluble in fat solvents. It has been called noctilucin by Phipson (6 and 7), photogen by Molish (8), luciferin by Dubois (9), and photogenin by myself. Phipson and Molish did not recognize that in many forms two sub-

[1]This paper is a compilation with additions and some changes of my work on luminous forms during the past three years. The original papers will be found grouped together in the bibliography (1), and are not specifically referred to in the text.

stances are concerned in light-production, but Dubois (9) pointed out that in the elaterid beetles and in the mollusc *Pholas dactylus* such was the case and he showed that these substances could be separated by heat. I can confirm this statement for certain other forms (lampyrid beetles and *Cypridina*), but find that the substances in *Cypridina* have many characteristics different from those described by Dubois for *Pholas* and that the mechanism of light-production is quite different from that which Dubois (10) supposes.[1] A record of the experiments and a discussion of the chemistry of light-production in all its aspects will be found in the following pages. *Cypridina*, as the most favorable species, will be considered first and then the other forms which I have studied—the luminous beetles, the pennatulid *Cavernularia*, luminous bacteria, the squid *Watasenia scintillans*, and a fish (*Monocentris japonica*)—together with a consideration of certain chemiluminescent reactions which involve the taking up of oxygen.

Most of the studies reported herein were made in Japan, a region unusually rich in luminous forms, through the kindness of Dr. Alfred G. Mayer and under the auspices of the Department of Marine Biology of the Carnegie Institution of Washington.

The success of my investigations was largely due to the interest and kindness of Professor C. Ishikawa, of the Zoology Department, Agricultural College, Imperial University of Tokyo, and to Professor I. Ijima, of the Imperial University, who extended to me the hospitality of the marine biological laboratory at Misaki. I am also indebted to Dean Kozai and Professor Aso, of the Agricultural College, for the use of many chemicals and apparatus. I extend my cordial thanks to all of these men.

STUDIES ON THE OSTRACOD CRUSTACEAN C. HILGENDORFII.

GENERAL CHARACTER OF LIGHT AND OF LUMINOUS GLANDS.

Cypridina hilgendorfii is a strongly negatively heliotropic ostracod best obtained at night by lowering into the water fish heads, on which the animals feed. It may be caught the year round, but is most abundant during August and September in Sagami Bay, Japan. Another non-luminous species (*Cypridina x.*) is often obtained from the fish heads together with *C. hilgendorfii*. It is positively heliotropic to lamplight. *C. hilgendorfii* is so strongly negatively heliotropic that it is not readily caught on moonlight nights and avoids a shore with many electric lights.

The light-giving material of *Cypridina* is a secretion formed in a special gland of yellow spindle-shaped cells, opening near the mouth.[2]

[1] All of Dubois's papers are listed in Ann. d. la Soc. Linn. de Lyon for 1913. A general account is given in "La Vie et la Lumière" Alcan, Paris.

[2] For a description of the anatomy of the organ see a paper by N. Yatsu in Journ. Morph., 1917, and Dahlgren (19).

In adult living cypridinas the gland-cells form a cylinder about 0.64 mm. by 0.24 mm., having a volume of about 0.0003 c.c. The secretion is readily formed upon agitation of the animal and is easily visible in a room in the daytime. To the dark-adapted eye the color is decidedly bluish and the light very intense. To a partially light-adapted eye the color is blue. In this respect the light differs markedly from the yellowish white (to the dark-adapted eye) light of luminous bacteria of *Cavernularia* or of the Japanese firefly, which is green to the partially dark-adapted eye. Lund (3) describes a *Cypridina squamosa* (?) and a *Cyclopina gracilis* from Montego Bay, Jamaica, which gave an "intensely luminous greenish yellow secretion." Most observations, however, record the light to be decidedly bluish. There is no inhibition of the light secretion in daylight, as has been described for other forms (4 and 5). When first caught, cypridinas give off the secretion readily, sometimes upon mere contact with the surface film of water, but if they are kept in the laboratory the secretion is not given off so readily. Removal from sea-water also inhibits the ejection of the secretion and it is necessary to squeeze the animal rather strongly before the secretion is given out. Electrical stimulation also calls forth the production of an abundant light-secretion.

NATURE OF THE LIGHT-SECRETION.

The gland-cells are almost completely filled with yellow material which can be observed to be composed of yellow globules 2 to 6 micra in diameter. These are extruded and dissolve to a colorless solution, absolutely free of visible granules, which gives the light. The globules are rather fluid in consistency, as the outline can sometimes be seen to pass through amœboid-like changes.

If we examine the natural secretion of *Cypridina* in sea-water at night, the light appears perfectly homogeneous. No points of light appear, such as might be due to granules, a condition wholly different from that in the juice of *Cavernularia*, which contains visible granules, and at night the light from these under the microscope gives the appearance of a starry sky.

If cypridinas be ground in a mortar and then examined at night, numerous very bright points of light appear, and these are greatly increased by the addition of fresh water to the preparation. The light undoubtedly comes from solution of the above-described yellow globules and when they have dissolved no more points of light, but a steady homogeneous glow, is to be observed. Electrical stimulation by strong interrupted induced shocks does not intensify the light from an extract of cypridinas, glowing faintly.

PORCELAIN FILTRATION.

Although no visible granules are found in the natural secretion of *Cypridina*, an ultramicroscopic investigation was not undertaken on account of lack of apparatus.

All of the light-producing substances (both photogenin and photophelein, p. 178) will easily pass a Pasteur-Chamberland or a Berkefeld filter tube, so that the particles present, if any, must be exceedingly small.

Complete proof of the truly soluble character of the light-producing substances is given by dialysis experiments recorded on page 183.

LACK OF OXYGEN.

Oxygen is necessary for light-production, as may be seen by placing the crushed animals in a hydrogen atmosphere, or by bubbling hydrogen through a glowing extract of the animals. The light never completely disappears, even after a long time, but remains dim, so that very little oxygen (as no special precautions were taken to remove the last traces of oxygen from the hydrogen, prepared in a Kipp generator) is sufficient to give light. On readmitting oxygen, however, a brilliant glow results. Unlike the firefly (p. 196), an extract of *Cypridina* can be kept in absence of oxygen for over ten days with no light-production, but light again instantly appears if oxygen is readmitted.

DESICCATION AND ETHER EXTRACTION.

Cypridinas may be dried over $CaCl_2$ and will give a brilliant light if crushed and moistened with sea-water. Dried crushed cypridinas may be extracted with six changes of ether during the course of two days without impairing in the least their power to produce light when again moistened. The luminous substance is therefore of a non-lipoid ether-insoluble nature, as might be expected from the fact that it is extruded from the animal as a clear water-soluble non-fluorescent secretion.

CHEMICAL TESTS ON THE SECRETION.

Despite the fact that the light from the natural secretion of *Cypridina* is very bright, a sample of the secretion, collected by shaking many cypridinas in a small volume of sea-water and filtering, responds to none of the common biochemical tests. It gives no precipitate with picric acid or on saturation with $(NH_4)_2\ SO_4$ or on boiling, even when made slightly acid. Fehling's reaction is negative, as also the biuret and xanthroproteic for proteins, the Molish reaction for carbohydrates, and the indophenol test (*a*-naphthol and para-phenylen-diamine) $+ H_2O_2$ for oxidases.

I do not mean to infer from this that the luminous substance is neither protein, fat, or carbohydrate, but merely that the concentration giving a bright light is too small to respond to chemical tests (see p. 188).

LUCIFERIN AND LUCIFERASE.

The light from the natural secretion of *Cypridina* lasts for some time in sea-water and then disappears, and no amount of shaking will cause

it to appear again. If we add to this natural secretion an extract of *Cypridina* heated to boiling, a brilliant light again results; or if we mix a water extract of *Cypridina* whose light has disappeared on standing with a similar extract whose light has been destroyed by boiling, light again results. As already mentioned, Dubois first demonstrated this for *Pyrophorus* and a year later for *Pholas dactylus.* In Dubois's phraseology we have mixed two substances—luciferin (in the boiled tube) and luciferase (in the tube allowed to stand)—necessary for light-production. According to Dubois (10), one of these, luciferase, is an oxidizing enzyme and is destroyed by heat; the other, luciferin, a substance not destroyed by heat, is capable of oxidation with light-production by means of luciferase. When the natural secretion of *Cypridina* is allowed to stand all the luciferin is oxidized and the luciferase is left; when a luminous extract of *Cypridina* is boiled the luciferase is destroyed before all the luciferin is oxidized. The two substances alone in solution are non-luminous.

At one time I believed Dubois's interpretation of this experiment to be correct, but results on *Cypridina* have led me to wholly different conclusions regarding the existence of luciferin and luciferase. Dubois's interpretation is indeed attractive. We know that the light-production is an oxidation; that two substances are concerned; that these substances give light in very small concentration comparable with enzyme activity (see p. 188); that one of them can use up a large amount of the other (see p. 189) and possesses certain properties (destruction by heat, phosphotungstic and tannic acid) characteristic of enzymes. Further, we actually know of oxidative reactions taking place with the production of light under the action of oxidizing enzymes from plants and animals (see p. 225).

It is quite possible that light-production in *Pholas dactylus* is of this nature, as it differs radically in very essential points from the mechanism in *Cypridina* and in the firefly. Thus, Dubois finds that *Pholas* luciferin (the substance not readily destroyed by heat and giving light with luciferase) can be oxidized with light-production by many oxidizing agents—blood, H_2O_2, $KMnO_4$, BaO_2, PbO_2, etc.—and that it occurs only in the luminous organs of *Pholas dactylus* (10). I find that *Cypridina* luciferin (a substance not readily destroyed by heat and giving light with luciferase) will not give light with the above-mentioned oxidizing agents and is found in many other non-luminous animals and in the non-luminous parts of *Cypridina hilgendorfii.*

Dubois finds that *Pholas* luciferase (a substance destroyed easily by heat and using up *Pholas* luciferin with light-production) occurs in many other non-luminous animals (10), whereas I find a body with the same properties only in the luminous organs of *Cypridina.* This substance, which we may temporarily call *Cypridina* luciferase, in concentrated solutions, will give light (as before mentioned) with extracts of

many non-luminous animals. It will also give light if mixed with many pure substances, as chloroform, ether, benzol, thymol, saponin, oleic acid, atropin, NaCl, and others. Since most of the above substances could not possibly be oxidized by the luciferase, I conclude that they cause in some way the giving out of light in what Dubois terms luciferase. On this view luciferase is the source of the light and the luciferin (in the boiled extract of *Cypridina*) is something which causes the luciferase to emit light.

The substances causing the emission of light from a concentrated solution of luciferase are similar to those producing cytolysis of cells, and I have considered the possibility that the concentrated extract might contain fragments of the luminous gland-cells which cytolyze with light-production or possibly granules which dissolve with light-production, as described in many other forms, and as especially prominent in the juice of *Cavernularia*. I am, however, convinced that there are in the extract of cypridinas which will give light with unoxidizable substances no cell fragments present and no granules above those of submicroscopic colloidal dimensions, for the following reasons:

First, the light is always perfectly homogeneous and in marked contrast to the points of light of *Cavernularia* juice, where visible granules and cell fragments do occur.

Second, *Cypridina* extract (luciferase) will give light with thymol, butyl alcohol, or NaCl crystals after filtration through a Chamberland or a Berkefeld filter, which removes all cell fragments.

PHOTOPHELEIN AND PHOTOGENIN.

I conclude, therefore, that Dubois's luciferase, a thermolabile substance in luminous cells, is the light-producing body. It gives light in contact with many substances not necessarily oxidizable and an especially bright light with a thermostabile substance (Dubois's luciferin) found in high concentration throughout the body of *Cypridina hilgendorfii* and in small concentration in the blood or juices of other non-luminous invertebrates. I therefore propose the new names photogenin (light-producer, from φῶς, light, and γεννάω, produce) for luciferase and photophelein (light-assister, from φῶς, light, and ὠφελέω, assist), for luciferin.

As we shall see later (p. 189), the photophelein can not be regarded as an enzyme, because it is used up in the reaction and will not give light with an indefinite amount of photogenin. The photogenin corresponds much more to an enzyme, but it is also slowly used up. At present we can only speak of photophelein as a definite substance causing in some way the emission of light from photogenin.

Perhaps the comparison of the important facts concerning light-production in *Pholas* and *Cypridina* shown in table 1 will make clearer Dubois's and my own views, and bring out the contradictory properties

of the corresponding substances in the two animals. A more complete table is given on page 200.

Whatever the exact interpretation of the facts may be, it is certain that two substances are concerned in light-production, and these may be separated, because they are destroyed at different temperatures. We may now inquire into each of their properties separately and return to a discussion of the mechanism of light-production, in considering the possible enzyme nature of photogenin.

TABLE 1.

	Pholas.		Cypridina.	
	Luciferin (oxidizes with light-production by luciferase).	Luciferase (enzyme accelerating oxidation of luciferin).	Photophelein (substance assisting in production of light by photogenin).	Photogenin (auto-oxidizable in presence of photophelein).
Destruction by heat	−	+	−	+
Dialyzable	+	−	+	−
Stability	+	−	−	+
Occurrence in non-luminous parts	−	+	+	−
Oxidized with light-production by $KMnO_4$, H_2O_2, etc	+	−	−	−

DISTRIBUTION OF PHOTOPHELEIN AND PHOTOGENIN IN ORGANISMS.

Certain fundamental facts for the chemical theory of light-production appear when we study the distribution of photophelein and photogenin: (I) in the non-luminous parts of *Cypridina*; (II) in the non-luminous *Cypridina*; (III) in other non-luminous organisms; (IV) in luminous organisms.

By a careful, quick scissors-cut, the head end of *Cypridina* containing the luminous gland can be separated from the posterior half without any contamination of the latter with luminous secretion. If we now test the non-luminous half with dilute photophelein and photogenin, we find that it contains nothing which will give light with photophelein, but something which will give a bright light with photogenin. We must try the experiment immediately, because this substance disappears if the extract stands in presence of oxygen. In absence of oxygen, or if the extract is boiled immediately (but not too long a time), the substance does not completely disappear, even after an hour. There is, therefore, in the non-luminous parts, the substance photophelein, which disappears even in the absence of photogenin (from luminous gland), unless the solution be boiled or oxygen excluded. (Note the

similarity to the disappearance of photophelein in the presence of photogenin, with this exception, that no light is produced.) The experiment seems to indicate a third substance destroyed by heat, in non-luminous parts, which oxidizes (since oxygen is necessary) the photophelein.

In the extract of the non-luminous species, there is also a similar substance (photophelein) which will give light with photogenin. Unlike the photophelein from the non-luminous part of *Cypridina hilgendorfii*, it occurs in very small concentration, so that we must use concentrated[1] photogenin and concentrated extract of *Cypridina x*.

Table 2.

Animal.	Order.	Character of light from juice.	
		Unboiled.	Boiled.
Sepia esculenta (blood)	Cephalopoda	Fair	Fair.
Dolabella sp. (blood)	Gasteropoda	Faint	Fair.
Laonome japonica (blood)	Annelida	Very faint	Very faint.
Panulirus japonica (blood)	Decapoda	Very faint	Faint.
Lepas anatifera (extract)	Cirripedia	Good	Bright.
Tetraclita porosa (extract)	Cirripedia	Faint	Fair.
Mitella mitella (extract)	Cirripedia	Faint	Fair.
Oxyrhynchidæ (moss crab) (extract)	Decapoda	Faint	Fair.
Palæmon sp. (extract)	Decapoda	Faint	Faint.
Chiton (extract)	Amphineura	Fair	Good.
Hormithoe imbricata (extract)	Annelida	Fair	Fair.
Cirratulus dasylophius (extract)	Annelida	Very faint	Very faint.
Marphysa iwamusi (extract)	Annelida	Faint	Faint.
Onchidium sp. (extract)	Gasteropoda	Faint	Faint.
Non-luminous parts firefly (extract)	Insecta	Negative	Fair.
Flat worm (sp.?) (extract)	Turbellaria	Faint	Faint.

The photophelein from *Cypridina x* is also destroyed or disappears if the extract stands $1\frac{1}{2}$ hours in contact with oxygen, but not in the absence of oxygen. Boiling makes the extract more stable.

In some other non-luminous forms, widely different in relationship from *Cypridina*, there are substances which give light with concentrated photogenin and others which will not, whether the extract has been boiled or left unboiled. The extracts were boiled in order to destroy substances which in turn might quickly destroy photophelein.

Among the light-giving extracts may be mentioned those listed in table 2.

Of these forms, *Lepas* and *Chiton* gave the best light and of these two only *Lepas* gave light with dilute *Cypridina* photogenin. Too much stress must not be laid upon comparative results, because much depends upon the concentration and it is not easy to obtain extracts of comparative concentration.

[1]By concentrated photogenin is meant an extract of 1 *Cypridina* in 0.5 to 1.0 c.c. water.

Unlike the photophelein from the luminous parts of *Cypridina* or from non-luminous cypridinas, that of *Lepas* extract or of *Dolabella* blood is perfectly stable and will give light with photogenin even after standing for a period of 24 hours.

Many extracts were found to give no light with concentrated photogenin. These included those shown in table 3, which were tried both boiled and unboiled. Again it is possible that with greater concentrations even these extracts would call forth a faint light.

The following fluids and dissolved protein substances also give no light when their solutions are mixed with photogenin: 50 per cent egg albumen; 50 per cent egg yolk; Na nucleate; Na nucleoproteinate; Witte's peptone; neutral meat peptone; dried ox-blood extract.

TABLE 3.

Animal.	Order.	Animal.	Order.
Modiolus sp.?	Lamellibranch.	Ligia exotica	Isopod.
Acmæa sp.?	Gasteropod.	Coccinella 7-punctata	Coleoptera.
Sistrum sp.?	Gasteropod.	Anomala rufscuprea	Coleoptera.
Sphærechinus pulcherrimus.	Echinoid.	Glysiphana jocunda	Coleoptera.
Wall crab (sp.?)	Decapod.		

It is certain, then, that there is photophelein or something similar to it in the blood or extracts of many invertebrates, but not necessarily in solutions of protein substances such as egg albumen, peptone, etc. In saliva there is something giving a very faint light and something in urine giving a fairly bright light with photogenin. Certain fluids tried were sufficiently acid to prevent the appearance of light and some fortuitous characteristic, such as acidity, may explain why extracts of some invertebrates give no light with photogenin.

It is hardly worth inquiring into the nature of the substances in each particular extract which may for convenience be collectively spoken of as photophelein, since I have found a great many simple bodies which, mixed with concentrated photogenin in powder or crystal form, give rise to a bright light. With more dilute solutions (one *Cypridina* to 10 c.c.) no light appears. These substances include sodium glycocholate, sodium taurocholate, thymol, chloroform, hydrochinon, NaCl, butyl alcohol, saponin, oleic acid, ether, benzol, atropin, pilocarpin, and ortol. Chloral hydrate, pyrocatechin, $FeSO_4$, $MgSO_4$, $(NH_4)_2SO_4$, esculin, dextrine, and $K_4Fe(CN)_6$ gave a fainter light, while saccharose gave none.

It is difficult to see in just what way all these diverse substances act. One is inclined to compare the production of light by chloroform or saponin to a process of cytolysis or to a stimulus reaction like artificial parthenogenesis. The photophelein of *Cypridina* or other extracts would then be comparable to the cytolytic substance in the blood of

worms which causes development of sea-urchin eggs (11). The chloroform or saponin might be supposed to cause the solution of granules or globules (as can be observed in many cells) and the production of light to be connected with this solution process. But I have already given evidence (p. 178) to show that there are no granules unless ultramicroscopic ones in the light-secretion of *Cypridina*, so that we must look to another interpretation.

Of many animal extracts tried on both concentrated and dilute photophelein, none will give light. Neither have I been able to obtain light with any chemicals or the oxidizing substances—neutral H_2O_2, BaO_2, ox blood alone or with neutral H_2O_2 added, neutral potato juice alone or with neutral H_2O_2, $KMnO_4$, $K_2Cr_2O_7$, $FeCl_3$, or $K_4Fe(CN)_6$. As already pointed out (p. 177), this result is the direct opposite of the case in *Pholas dactylus* as described by Dubois.

There is, then, in the non-luminous parts of *Cypridina hilgendorfii* a large amount of photophelein, but only a small amount even in a closely related non-luminous form. There appear to be only small amounts in other luminous forms not closely related to *Cypridina*. For instance, *Cypridina* photogenin will give a faint light with firefly photophelein, but it is not nearly so bright as with *Cypridina* photophelein nor as bright as that given by non-luminous *Lepas* extract. Unfortunately, no closely related luminous crustacean was available, so that the degree of specificity of photogenin and photophelein could not be determined. At least we may say that *Cypridina* photogenin and photophelein are specific to a certain extent, but not in the strict sense of the word.

The following tabulation gives the luminous animals which have been tried with *Cypridina* in concentrated solution and the character of the light resulting. None gives light in more dilute solution.

Cypridina photophelein × firefly photogenin	Faint.
Firefly photophelein × *Cypridina* photogenin	Faint.
Firefly photophelein × firefly photogenin	Bright.
Cypridina photophelein[1] × *Cavernularia* photogenin	Fair.
Cavernularia photophelein × *Cypridina* photogenin	Negative.
Cavernularia photophelein × *Cavernularia* photogenin	Negative.
Cypridina photophelein × *Noctiluca* photogenin[2]	Faint.
Noctiluca photophelein[2] × *Cypridina* photogenin	Faint.
Noctiluca photophelein[2] × *Noctiluca* photogenin	Negative.

Owing to lack of material, the luminous fish *Monocentris japonica* could be tested only in more dilute solutions, and with negative results, viz:

Cypridina photophelein × *Monocentris* photogenin	Negative.
Monocentris photophelein × *Cypridina* photogenin	Negative.
Monocentris photophelein × *Monocentris* photogenin	Negative.

[1]An extract prepared with sea-water, as the dark juice of *Cavernularia* gives a brilliant light with fresh water.

[2]All the *Noctiluca* extracts were made with n/1000 NH_4OH to neutralize the acid of the *Noctiluca* vacuoles.

DIALYSIS.

Photophelein will dialyze through heavy parchment and collodion fairly readily, in the case of collodion sometimes appearing in the dialysate in the course of 2 hours.

Photogenin dialyzes with difficulty and with some collodion tubes not after a period of 36 hours. In others a very slight amount will pass in that time and in one experiment with heavy parchment paper a very slight dialysis occurred in 12 hours, but usually there was none. The collodion tubes and the paper did not leak in any of the experiments recorded.

ADSORPTION.

Both photophelein and photogenin are removed from solution by washed boneblack and washed freshly precipitated $Fe(OH)_3$. To serve as control the last washings from boneblack and $Fe(OH)_3$ were added to photogenin and photophelein and tested with photophelein and photogenin, respectively, to make sure that the adsorption was not apparent and due to destruction by foreign substances from the adsorbing media.

TEMPERATURE.

Lund (3) finds that in *Cypridina squamosa* (?) and *Cyclopina gracilis* the light from the luminous secretion disappears at 50° and if heated above 50° (as high as 70° but not above) it will again reappear at 50°. The time factor is always involved in determining the critical points in the effects of temperature, and I find that the concentration of the light-producing substances is also involved. Therefore the exact temperature of destruction of photephelein and photogenin depends upon their concentration and the time of heating. I find that cypridinas dried over $CaCl_2$, ground, and the powder suspended in sea-water give a beautiful light which disappears when heated to 56°, but returns on cooling. If heated to 65° and cooled, the light also returns, but does not return if heated to 70° and then cooled. A very concentrated mixture of photogenin and photophelein may be heated above 70° and whole cypridinas heated to boiling will occasionally give a faint light when cooled. The light from the normal secretion of *Cypridina* disappears at 52° to 54°, and returns on cooling, so that we may regard this as the inhibition temperature and something above 70°, depending on the concentration, as the destruction temperature. These results are in good agreement with those of Lund.

The time necessary to destroy photophelein at 100° depends also upon its concentration. In dilute solution (1 *Cypridina* to 25 or 50 c.c. water) boiling for 1 minute is sufficient, but in concentrated solution (1 *Cypridina* to 1 c.c. water) 5 minutes' boiling is necessary.

In *Pholas dactylus* (5) photophelein (luciferin) is destroyed above 70°, whereas in the firefly (both *Photuris* and *Luciola*) it may be boiled for 10 minutes without destruction.

The luminous material of *Cypridina*, like that of most luminous organisms, is unaffected by cold and will glow brilliantly at 0° C.

STABILITY.

Cypridina photogenin is much more stabile than photophelein. The time after preparation that one can obtain light from these substances depends largely upon their concentration, as also upon the temperature. One sample of concentrated (1 *Cypridina* to 2 c.c.) photogenin gave light with fresh photophelein for 7 days at 20°, despite the fact that decomposition had taken place and the liquid smelled foul. Dilute photogenin (1 *Cypridina* to 50 c.c.) may lose its power in 1 day; very dilute photogenin (1 *Cypridina* to 12,500 c.c.) in less than 2 hours.

Cypridina photophelein also disappears from solution spontaneously, and the more dilute the solution the more quickly does it lose its power to phosphoresce. With one *Cypridina* to 25 c.c. the power disappears in 3 to 4 hours, while with 1 *Cypridina* to 1 c.c. it may last for 5 days at 26° to 28° C.

The decomposition occurs whether the solution be neutral, acid (n/8000 HCl), or alkaline (n/4000 NaOH), in pure-water or sea-water, in the light and also in amber bottles, and occurs even if the photophelein be thoroughly boiled to destroy all traces of photogenin which might slowly use up the photophelein without light-production (see p. 189).

The photophelein is apparently oxidized, as the destruction is not so rapid in absence of oxygen.

TABLE 4.—*Effect of preservatives on photophelein.*

Saturation with—	Tested with photogenin after—			
	5 minutes.	1 hour.	1 day.	2 days.
Ether	Bright light	Negative	Negative	Negative.
Benzol	Bright light	Very, very faint	Negative	Negative.
Chloroform	Bright light	Very faint	Negative	Negative.
Thymol	Bright light	Faint	Negative	Negative.
Control	Bright light	Bright light	Faint[1]	Negative.

[1]Often photophelein will not last for one day.

PRESERVATIVES AND ANESTHETICS.

The addition of preservatives (anesthetics), as chloroform, ether, benzol, and thymol, is to hasten the spontaneous destruction of the photophelein and to preserve the photogenin.

Ether is especially destructive to the photophelein, whereas photogenin will give light after saturation with ether for 22 days. Tables 4 and 5 show the effect of saturation of solutions (1 *Cypridina* to 25 c.c.) of photophelein and photogenin with the four substances.

The harmlessness of the above anesthetics for *Cypridina* photogenin is unusual, as Dubois found a marked destructive action on *Pholas* photogenin (luciferase) and I have noted the same thing for the firefly.

As we have just seen, the addition of certain anesthetics does not rapidly destroy photophelein or photogenin. We can saturate a phosphorescent mixture of the two with ether and the light will still last for some time. If we add butyl alcohol to saturation the light disappears, and if the solution is now diluted with water or sea-water, the light reappears. The same phenomenon is observed if the photogenin be filtered through a Chamberland porcelain filter to remove all traces of cells or cell fragments. Care was taken to make sure that the return of light was not due to fluid adherent to the sides of the test-tube and untouched by the butyl alcohol.

TABLE 5.—*Effect of preservatives on photogenin.*

Saturation with—	Tested with photophelein after—					
	1 day.	2 days.	7 days.	22 days.	50 days.	130 days.
Ether	Bright...	Bright...	Faint.....	Very faint.	Negative.	
Benzol	Bright...	Bright...	Bright....	Negative.		
Chloroform	Bright...	Bright...	Bright....	Bright....	Bright....	Negative.
Thymol	Bright...	Bright...	Faint.....	Faint.....	Negative.	
Control	Bright...	Faint....	Negative.			

A similar phenomenon is observed with ethyl alcohol and acetone. If we add in small amounts absolute ethyl alcohol to a glowing mixture of photogenin and photophelein, the light becomes very dim when 16 per cent alcohol has been added and disappears with 20 per cent alcohol. If now the mixture be diluted, the light returns. Acetone[1] behaves as alcohol. About 23 per cent is necessary for extinction of the light. Saturation with chloretone does not extinguish the light.

The effect of ethyl alcohol and acetone might be explained as the effect of precipitation, because of insolubility in the 20 per cent solution, but we can not so explain the extinction of the light by butyl alcohol and subsequent recovery on dilution, since butyl alcohol is only soluble to the extent of 8.3 parts in 100 parts of water. We are dealing with a highly interesting effect—one akin to anesthesia—a reversible inhibition, not of a cell or cell fragments, but of a solution. Filtration through porous porcelain shows that no cell fragments can be present. I can not here enter more fully into a discussion of this interesting phenomenon, whose bearing on anesthesia is obvious, except to point out that if we can anesthetize a solution we need not, as some recent theories have done, regard changes in the cell-membrane to be necessarily the ultimate cause of anesthesia.

It may be pointed out in passing that the production of light gives us the opportunity of observing the effect of, let us say, an anesthetic

[1]The sample of acetone at hand was not particularly pure.

or temperature upon a process at any particular instant, rarely obtained in the study of enzyme action or even of cell action, except where some movement is affected. For instance, we can heat pepsin to 60° and then cool it and see if it will digest protein. If we find that it will digest protein, that gives us no information as to a reversible inhibition at 60°, an inhibition which might become non-reversible if we kept the pepsin at 60° long enough to test directly the power of digestion. We can, however, heat photogenin and photophelein to 60°, cool them, and find out immediately if they give light.

PROTEIN PRECIPITANTS.

The light disappears in a phosphorescent mixture of photogenin and photophelein when the following substances are added to the concentrations indicated: tannin—m/2500 to m/5000; phosphotungstic acid—m/2500 to m/5000; picric acid—m/500, assuming a saturated water solution to be 1.03 per cent or m/22.

TABLE 6.—*Effect of acids and alkalies on light-production.*

Concentration in mixture.	Character of light.
n/250 NaOH	Instantly disappears.
n/500 NaOH	Instantly very dim, lasting >5 minutes.
n/1000 NaOH	Instantly dim, lasting >5 minutes.
n/2000 NaOH	Instantly fair, lasting >5 minutes.
n/4000 NaOH	Instantly fair, lasting >5 minutes.
n/8000 NaOH	Instantly fair, lasting >5 minutes.
Control	Remains good >5 minutes.
n/2000 HCl	Instantly disappears.
n/4000 HCl	Instantly dim, lasting >5 minutes.
n/8000 HCl	Remains good >5 minutes.

A peculiar phenomenon observed with phosphotungstic and tannic acids but not with picric acid is that, at the lower limit of extinction, the light reappears after first disappearing and more acid must be added in order to extinguish it again. Care was taken to make sure that the phenomenon was not due to more perfect adaptation of the eyes to the dark. When the light is extinguished by dilute picric acid it will reappear again on dilution with water.

ACIDS AND ALKALIES.

The effect of acids (HCl) and alkalies (NaOH) can be seen by adding 1 c.c. of the acid (or alkali) of a definite concentration to 1 c.c. of a glowing mixture of photogenin and photophelein. The concentration of the acid or alkali in the mixture will thus be one-half of the concentration added. The results are given in table 6.

The effect of acid (HCl) and alkali (NaOH) was also studied in the following way: 1 c.c. fairly dilute (1 *Cypridina* to 10 c.c.) photophelein was mixed with 1 c.c. n/500 HCl, allowed to stand 1 hour and then

neutralized by adding an equal volume of n/1000 NaOH and tested for light by adding photogenin. The photophelein was therefore in contact with n/1000 HCl for 1 hour. In this way the effect of acid on photogenin and photophelein can be separated. The results are given in table 7.

TABLE 7.—*Effect of acid and alkali on photophelein and photogenin.*

Concentration of acid and alkali.	Effect on photophelein neutralized after 1 hour and photogenin added.	Effect on photogenin neutralized after 1 hour and photophelein added.
Concentration of HCl:		
n/1000	Negative	Negative.
n/2000	Very faint	Negative.
n/4000	Bright	Very faint.
n/8000	Bright	Bright.
Control in water	Bright	Bright.
Concentration of NaOH:		
n/31	Faint	Negative.
n/62	Faint	Negative.
n/125	Faint	Negative.
n/250	Faint	Faint.
n/500	Faint	Fair.
n/1000	Faint	Fair.
Control in water	Good, faint	Fair.

Even very small concentrations of NaOH added to a glowing mixture of photogenin and photophelein will decrease greatly the amount of light (table 6). Thus if we add 1 c.c. n/500 NaOH to 1 c.c. of a brightly glowing mixture, making the concentration of NaOH n/1000, the light instantly becomes very dim and remains so for a considerable time. If NaOH be added to a concentration of n/250, the light disappears completely; if we now neutralize the NaOH with n/250 HCl, the light returns, faint.

Still smaller concentrations of HCl prevent light-production. Thus (table 6) n/2000 HCl extinguishes the light from a luminous mixture, but on neutralization with n/2000 NaOH, the light returns and is fairly bright. The effect of NaOH and HCl is therefore to inhibit light-production, and not to immediately destroy the photogenin and photophelein (tables 6 and 7). The reversibility is not apparent if the acid or alkali are only slightly stronger than the values given. (Note also from table 7 that the photogenin is more readily affected by HCl and especially by NaOH than the photophelein.)

In the case of acid and alkali, as with so many other substances on the light-giving material, the effect is to an extent reversible if the substance is removed. The concentration of HCl which inhibits light-production is the same as that affecting cells, although the concentration of NaOH necessary to inhibit light-production is somewhat greater than that necessary to affect cells (12 and 13).

POTASSIUM CYANIDE.

The effect of KCN is of especial interest because of its power of inhibiting cell oxidations. It was tested by mixing an equal volume of photogenin (1 *Cypridina* to 25 c.c.) with the KCN solution, and testing with an equal volume of photophelein (1 *Cypridina* to 12 c.c.) after 10 minutes and after 1 hour. Table 8 gives the results.

TABLE 8.—*Effect of KCN on light-production.*

Concentration of KCN in photogenin solution.	Light with photophelein after 10 minutes.	Light with photophelein after 1 hour.
n/20	Fair.	
n/30	Fair.	Fair.
n/125	Fair.	Fair.
n/250	Bright.	Bright.

In *Cypridina*, as in all other luminous animals which have been tried (*Cavernularia*, *Noctiluca* (2), firefly, luminous bacteria), KCN is practically without influence on light-production. *Cavernularia* juice, for instance, will light for over 90 minutes in n/40 KCN. On the other hand, n/1280 KCN is sufficient to completely inhibit the oxy-luminescence of pyrogallol by the vegetable oxidases (see p. 230).

SATURATION WITH SUGAR, $(NH_4)_2SO_4$, AND NaCl.

Saturation of a luminous mixture of photogenin and photophelein with sugar or NaCl causes the light to disappear and it reappears on dilution of the mixture with water.

Since the $(NH_4)_2SO_4$ used was acid, a small amount of this salt caused the light to disappear and it did not reappear on dilution of the mixture with water.

Probably these phenomena are connected with the salting-out of the luminous substances, although no signs of a precipitate are visible when the natural secretion of *Cypridina* is saturated with NaCl or $(NH_4)_2SO_4$. As already pointed out, this result is no doubt due to the small concentrations of the substances present.

CONCENTRATION OF PHOTOGENIN AND PHOTOPHELEIN IN CYPRIDINA.

In the normal secretion of *Cypridina* there is more photogenin than photophelein, as may be seen by adding fresh photophelein to the normal secretion after the light has disappeared on standing. The light again appears. The photophelein had been completely used up. This may be shown in another way by allowing a concentrated mixture of photogenin and photophelein to stand until the light disappears, and then boiling one-half of the mixture. Upon mixing the two halves no light results, as all the photophelein had been used up before the photogenin was destroyed in the tube boiled.

The concentrations of the two substances which will give visible light when mixed are very small. In one experiment performed 15 minutes[1] after the photophelein was obtained, 1 *Cypridina* in 25,600 c.c. of water gave a just-visible light when mixed with an equal volume of more concentrated photogenin. The photogenin from 1 *Cypridina* in 25,600 c.c. of water will also just give visible light when mixed with an equal volume of more concentrated photophelein.

We can see how small an amount of photogenin will give light when we consider that the animal is about 3.5 by 2.5 by 1.5 mm. and that the luminous gland is more or less of a cylinder 0.64 mm. high by 0.24 mm. diameter, with a volume of about 0.03 mm. or 0.00003 cm. and that 1 luminous gland in 25,600 c.c. water will give visible light when mixed with an equal volume of photophelein, or in a concentration of 51,200 c.c. Hence 1 part of gland in about 1,700,000,000 parts of water will give visible light upon the addition of photophelein. Even this is a low estimate, as we do not know what the concentration of the photogenin is in the gland itself.

When we compare the amount of photogenin which can be detected by light-production with the amount of substances detectable by chemical means, we see how extraordinarily sensitive the light-reaction is. AgCl is soluble in water to the extent of 1 part in 625,000 parts water at 20° (**14**), so that more chloride than this must be present to be detected by $AgNO_3$, a very delicate reaction. $KMnO_4$ is roughly just distinguishable by its color in a 14 mm. test-tube in a concentration of 1 to 1,250,000.

Biological reactions are, generally speaking, more delicate than inorganic reactions. Zinc in traces has a favorable influence on the growth of molds and 1 to 25,000,000 will increase the weight of a crop of *Aspergillus* by 50 per cent (**15**). Copper will kill *Spirogyra* in 1 minute when present in 1 to 77,000,000 concentration (**15**). According to Kastle (**16**), blood in 1 to 80,000,000 can be recognized by the phenolphthalein reaction. Since this reaction is due to hemoglobin, of which blood contains about 10 per cent, the hemoglobin can be recognized in 1 to 800,000,000 concentration. These figures give an idea of the delicacy of the photogenin-photophelein reaction as compared with others. It should be borne in mind that pyrogallol in 1 to 254,000 will give visible light with potato-juice and H_2O_2 (see p. 225).

IS PHOTOGENIN USED UP IN LIGHT-PRODUCTION WITH PHOTOPHELEIN?

The exceedingly small concentration of light substances which give visible light suggests that one or both are of enzyme nature, as Dubois supposes. There are two ways of testing this question. One is to

[1]The experiment was performed as quickly as possible, because the photophelein disappears on standing.

determine whether a small concentration of photogenin can use up a large concentration of photophelein, providing a sufficiently long time is allowed. We can not use this method, because photophelein decomposes spontaneously.

Another way is to determine if a small amount of photogenin can decompose successively added amounts of photophelein without itself undergoing diminution. This method is not unequivocal, since many true enzymes are paralyzed or destroyed by the decomposition products of the reaction which they accelerate.

It was found that if we add to 1 c.c. of a weak (1 *Cypridina* to 50 c.c.) solution of photogenin, successive 1 c.c. additions of a concentrated (1 *Cypridina* to 2 c.c.) solution of photophelein as soon as the light from the preceding addition has disappeared, after four 1 c.c. additions no more light will appear. The photogenin is therefore used up and can not oxidize additional photophelein, although there is plenty of photophelein present, as may be shown by adding fresh photogenin, when a good light appears. With each successive addition of concentrated photophelein, the light, which at first is very bright and lasts about an hour, becomes less brilliant and lasts a shorter time. This is not due to mere dilution of the dilute photogenin, as we can dilute the dilute photogenin to the same volume with water and then upon the addition of photophelein a good light results. We can conclude only that, although photogenin can use up a large amount of photophelein, it is itself changed in some way in the reaction and disappears. We can not say how much photophelein will combine with a definite quantity of photogenin, because we do not know the absolute amount of these substances in a single *Cypridina*. In the above experiment we added a concentration of photophelein from one *Cypridina* 100 times (*i. e.*, four additions each 25 times more concentrated) that of the photogenin from one *Cypridina*.

Although the evidence goes to show that the photogenin is used up, it is not nearly so rapidly used up as is the oxidase of potato in the production of light from pyrogallol (see p. 231). We must remember also that certain enzymes, as zymase (thermolabile and non-dialyzable), are only active in presence of a coenzyme which is not destroyed by heat and is easily dialyzable. In fact, the photogenin-photophelein system resembles to a very remarkable degree the zymase-cozymase system. There is the same quantitative relation between zymase and cozymase as between photogenin and photophelein. If zymase is present in excess, the coenzyme is all used up; if cozymase is in excess then the zymase is used up (17). Nevertheless, however much photogenin resembles cozymase, I have for the present deemed it best to avoid the termination *ase*. In absence of more definite knowledge we may provisionally regard photogenin as a substance auto-oxidizable only in presence of photophelein.

If we try the reverse experiment, that of adding 1 c.c. of concentrated (1 *Cypridina* to 2 c.c.) photogenin to 1 c.c. of dilute (1 *Cypridina* to 50 c.c.) photophelein, a bright light appears lasting 10 to 15 seconds and no more light upon adding additional concentrated photogenin. As might be expected, the small amount of photophelein is very rapidly used up by the large amount of photogenin.

The quantitative relations between concentration of photophelein and photogenin, duration of light, and brightness of light will be considered in a subsequent paper.

SUMMARY OF RESULTS FROM CYPRIDINA.

1. The luminous secretion of *Cypridina* comes from several spindle-shaped yellow gland-cells on the upper lip and is extruded to the sea-water as a perfectly clear granule-free non-fluorescent secretion. The light is homogeneous and bluish white in color.

2. If the luminous secretion stands, the light disappears, and if we now add an extract of cypridinas heated to boiling, the light again returns—*i. e.*, *Cypridina* gives a luciferin-luciferase reaction similar to *Pholas dactylus*, as described by Dubois.

3. Contrary to Dubois's theory, the luciferase is the source of the light, and not an enzyme causing light-production by oxidation of luciferin, because we can obtain light from luciferase by substances incapable of oxidation (NaCl, chloroform, ether, etc.). The new names of photogenin or light-producer for luciferase and photophelein or light-assister for luciferin have therefore been proposed.

4. Oxygen is necessary for light-production.

5. Both photogenin and photophelein will pass a Pasteur-Chamberland or Berkefeld filter easily.

6. Photophelein dialyzes readily through heavy parchment or collodion, photogenin with great difficulty or not at all, even after a period of 36 hours.

7. Both photogenin and photophelein are adsorbed by boneblack and $Fe(OH)_3$.

8. The light-producing substances may be dried and thoroughly extracted with ether without impairing their light-giving power.

9. Chemical tests on the natural light-secretion give negative results, since a very small amount of light-substance gives a bright light and at least 1 part of photogenin or photophelein in 1,700,000,000 parts water will give a visible light.

10. Photophelein occurs throughout the body of *Cypridina*, photogenin only in the luminous organ. Photophelein from non-luminous parts disappears (apart from photogenin) on standing, but not so readily if the extract has been boiled or in the absence of oxygen. In the firefly and some non-luminous beetles the photophelein disappears so quickly that it is best to make the extract with boiling water. In

a non-luminous species of *Cypridina* a photophelein occurs in small quantity with properties similar to that in *Cypridina hilgendorfii.*

11. Photophelein occurs in extracts (both boiled and unboiled) of many non-luminous organisms, in greatest quantity in *Lepas anatifera.* The photophelein of *Lepas anatifera* does not disappear on standing.

12. Pure protein solutions (peptone, Na nucleoproteinate, albumen, etc.) or dried mammalian blood do not contain photophelein, but urine contains a similar body.

13. While photogenin will give light with many substances known and unknown, photophelein will give light only with the photogenin of luminous organs.

14. *Cypridina* photogenin will give no brighter light with extracts (photophelein) of other luminous forms (*Luciola, Cavernularia, Noctiluca*) than with extracts of non-luminous forms, and *Luciola* photogenin will not give so bright a light with *Cypridina* photophelein as with boiled extracts of non-luminous insects. We must conclude that the two substances are not specific for luminous forms, although there is a certain amount of specificity, for photogenin gives the best light with photophelein from the same species.

15. Bright light can be formed by *Cypridina* at 0° C. Photogenin is destroyed above 70°, the temperature and time depending on the concentration; photophelein only after several minutes' boiling, the time depending also on the concentration. The natural luminous secretion ceases to light at 52° to 54°, but the light returns on cooling.

16. Photophelein is relatively unstabile and disappears on standing, the time depending on the concentration. Photogenin is much more stabile, but also disappears slowly.

17. The spontaneous decomposition of photophelein is retarded (perhaps prevented) by lack of oxygen, but hastened by addition of preservatives (chloroform, ether, benzol, thymol). Photogenin can be kept longer by addition of preservatives. One sample preserved with chloroform retained its power to give light with photophelein for over 56 days at room temperature.

18. Saturation with ether, chloroform, benzol, thymol, or chloretone does not affect the light from a mixture of photogenin and photophelein. Saturation with butyl alcohol or 20 per cent ethyl aclohol or 16 per cent acetone extinguishes the light, and if the mixture is diluted with water the light reappears. This phenomenon of anesthesia of a solution is given by photogenin filtered through porous porcelain plus photophelein and difficultly soluble butyl alcohol, so that it can not be due to the presence of cell fragments or to insolubility in the 20 per cent ethyl alcohol.

19. Picric acid, tannic acid, and phosphotungstic acid extinguish the light in very weak concentrations, and the light returns in the case of picric acid if the solution is diluted with water. In the case of

tannic and phosphotungstic acids, the light returns after first disappearing, even without diluting.

20. HCl between n/2000 and n/4000 and NaOH between n/250 and n/500 extinguish the light, and the effect is reversible upon neutralization. It can be shown that both HCl and NaOH affect the photogenin more readily than the photophelein and that the effect of NaOH especially is more readily reversible.

21. KCN does not inhibit light-production even in strong concentration.

22. Saturation with $(NH_4)_2SO_4$, NaCl, or cane-sugar extinguishes the light most readily in the order named, and the effect is reversible except in case of $(NH_4)_2SO_4$ (due to acidity).

23. In the natural secretion of *Cypridina* or in the whole animal there is always enough photogenin to completely use up the photophelein. The photogenin from one animal will use up a large additional amount (at least 100 times the concentration in one animal) of photophelein, but not an indefinite amount, so that photogenin is not a true enzyme in the strict sense of the word, unless it be an enzyme poisoned by its own reaction products. The photogenin-photophelein system resembles the zymase-cozymase system to a remarkable degree, but it is best for the present to regard photogenin not as an enzyme but only as a substance auto-oxidizable only in presence of photophelein.

STUDIES ON FIREFLIES.

DESICCATION AND EXTRACTION WITH FAT SOLVENTS.

The experiments recorded herein have been made during a period of two years on several different species of firefly—the American *Photuris pennsylvanica* and *Photinus pyralis*, the Japanese *Luciola vitticollis* and *L. parva*, and the West Indian "cucullo," *Pyrophorus noctilucus* and *P. havaniensis*. All the species are essentially similar in behavior, and in general the statements apply to all.

Contrary to the condition in *Cypridina*, the photogenic substance is burnt within the cell which forms it and is found there in the form of granules scattered through the cytoplasm. The histological structure of the luminous tissue has been well described by many observers [Townsend (18), Lund (3), Dahlgren (19), Vogel (43), Bongardt (44), Geipel (45), Williams (46)] and need not be considered here.

The old observation that firefly luminous tissue can be dried and ground up and will phosphoresce when water containing oxygen is again added, gives a simple chemical method of investigating the nature of the photogenic material. The dried material may be extracted with water-free solvents (since the photogen does not oxidize in absence of water) and extracted material as well as the residue from evaporation of the filtrate may be tested for phosphorescence by adding water. The results indicate that a large number of fat solvents will extract

nothing from the dried tissue and leave the photogenic material unharmed. Indeed, the material may be extracted with boiling ether for 24 hours without impairing its power to phosphoresce. Boiling alcohol does destroy the power to phosphoresce, and the nature of its action is discussed below. These results, as well as the previous experiments of McDermott (20 and 21) and Dubois (22), using fresh watery material, show conclusively that the photogenic substance is not a fat or fat-like body of any kind. The results are given in table 9, which also gives the time of extraction and the temperature.

TABLE 9.

Substance.	Temperature (in degrees).	Time (in hours).	Extracted material.	Extract evaporated in vacuo.
Ether (cold)	20	72	+	–
Ether (hot)	35	24	+	–
Chloroform (cold)	20	72	+	–
Chloroform (hot)	61	8	+	–
Ethyl alcohol (cold)	20	24	+	–
Ethyl alcohol (hot)	78.4	24	–	–
Ethyl alcohol and ether (equal parts) boiling	44	10	+	–
Carbon tetrachloride	20	48	+	–
Carbon disulphide	20	48	–	–
Acetone	20	48	+	–
Toluol	20	48	+	–
Amyl alcohol	20	48	Very faint[1]	–
Ethyl butyrate	20	48	Very faint[1]	–

[1]The material was washed with ether to remove the amyl alcohol and ethyl butyrate.

A plus sign indicates phosphorescence when water is added and a minus sign indicates no phosphorescence. Both the original extracted material and the residue of the filtered extract evaporated to dryness were examined. The results indicate not only that the photogenic substance is not a fat, but also not a lecithin. I am aware that the lecithins are difficult to extract *in toto* from the cell, but this can be accomplished by a mixture of hot ether and alcohol, and yet a mixture of hot ether and alcohol will extract nothing which will phosphoresce from the firefly powder. We may safely say that the photogen is not a lecithin.

Of all the solvents tried, only hot alcohol and cold amyl alcohol and ethyl butyrate gave results that would indicate a possible solution of the photogenic substance; and yet there is nothing in the evaporated filtrate that will phosphoresce when water or a neutralized 3 per cent solution of H_2O_2 is added. Thinking that a second substance might be necessary and that this had not been extracted by the fat solvents, although the photogen had, the filtrate was also tested by adding a water extract of firefly organs, fresh or preserved with toluol or chloro-

form, and also by potato-juice which contains considerable quantities of oxidizing enzymes. In no case was phosphorescence observed. The boiling ethyl alcohol,[1] cold amyl alcohol, and ethyl butyrate must, therefore, break up the photogen. It is the alcohol itself and not the temperature (78.4°) of boiling alcohol which is responsible for the destruction of the photogen, as the dried powder will withstand this temperature for 24 hours without any appreciable diminution in its power to phosphoresce. McDermott finds that liquid sulphur dioxide and liquid ammonia also destroy the photogenic power (**20**).

EXTRACTION WITH AQUEOUS SOLVENTS AND EFFECT OF OXYGEN.

If the luminous tissues of the firefly are extracted with water a solution which still gives light on filtration through filter paper is obtained. The light lasts a certain time and then disappears.

If the dried powdered luminous tissues of the firefly are allowed to come in contact with oxygen-free water, no light is produced, but if we admit oxygen, in a few minutes we find the mixture becomes luminous. If we wait for an hour or more before admitting oxygen, no light is produced. The apparatus shown in figure 1 is convenient for demonstrating this.

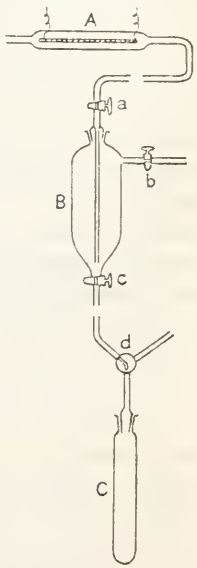


FIG. 1.—Apparatus for extraction of luminous tissue in absence of oxygen.

The material to be extracted is placed in the vessel *C* (fig. 1), provided with a ground-in stopper connected with a 120° stopcock. The water to be rendered free of oxygen is placed in *B* after passing hydrogen through stopcock *C* and closing it. *B* is connected through *A* with a hydrogen generator. The hydrogen is passed through potassium hydroxide to remove acid and then over a glowing platinum wire (in *A*) to remove the last traces of oxygen, a much better method than passing the gas through alkaline pyrogallol. By alternately exhausting *B* through *b*, connected to an air-pump, and refilling with hydrogen several times, the water can be quickly rendered free of oxygen. *C* is then connected to *B* through *c* and one of the arms of the 120° stopcock (*d*) whose other arm is connected with an air-pump. *C* and the arms of *d* are then exhausted. The 120° stopcock is then turned to connect *C* and *B* and *c* is opened, allowing the pressure of the hydrogen to drive the solvent on the material in *C*. The proper amount of fluid for extraction should be placed in *B*, so that the hydrogen may follow it through and fill the chamber *C*. Then *d* is closed, when *C* can be

[1]The 99.8 per cent absolute alcohol was distilled over metallic calcium and collected in a receiver protected from the air by $CaCl_2$ in order to remove the last traces of water.

disconnected and shaken during extraction. To filter the extract it is only necessary to connect one of the arms of *d* with a desiccator fitted with a funnel and filter rack. When the desiccator is exhausted, *C* and the desiccator are connected, and the pressure of the hydrogen in *C* drives the extract onto the filter paper. The firefly photogen begins to phosphoresce when the atmospheric pressure reaches 5 to 6 mm., which means an oxygen pressure of 1 to 1.2 mm. Consequently it is necessary to use a good vacuum-pump and make connections air-tight. I found small-bore lead tubing sealed with Khotinsky cement the best for the purpose.

All of my experiments have been carried out in the dark and the material observed at critical stages (as when the oxygen-free water was added) to make sure that no light appeared, and always with negative results. But to make sure that no very slow leakage of oxygen into the filtering chamber occurred, I have carried out the extraction in a special tube provided with a capillary sealed off during the extraction. After extracting in this tube for $1\frac{1}{2}$ hours and admitting oxygen, no phosphorescence appeared. Thinking that possibly the photogen dissolved in the extracting fluid did phosphoresce, but only so faintly as to be invisible because distributed through a relatively large volume of extract fluid, the unfiltered extract was evaporated *in vacuo* to a small volume. This can be very easily done by placing the rubber tube from the vacuum-pump over the capillary onto the special tube, exhausting, and then breaking the capillary through the walls of the rubber tube to connect with the air-pump. Even when concentrated the extract gave no light on adding oxygen.

We must conclude that the photogen is destroyed in distilled water, even without oxidation. The search for watery solvent for the photogen becomes, then, a search for a solvent in which the photogen is stable. The following solutions were tried in addition to distilled water. Extraction was allowed to proceed for from 1 to $1\frac{1}{2}$ hours.

1. Ringer's solution (as representing fairly accurately the concentration and composition of the firefly's blood).
2. 0.125 m NaCl.
3. Sea-water (a mixture of chlorides and sulphates of Na, K, Ca, and Mg).
4. 5 per cent NaCl.
5. 0.05 m NaOH and 0.1 m NaOH. The dried, powdered firefly organs will phosphoresce strongly if sprinkled on the surface of 0.1 m NaOH.
6. 0.02 m HCl. Dried firefly powder will phosphoresce on 0.0125 m HCl and on 0.025 m HCl, but less brilliantly. Only one or two bright dots appear on 0.05 m HCl and no phosphorescence occurs on 0.1 m HCl. If neutralized within two minutes after contact with the acid, the light does not appear in the 0.1 m HCl treated material nor become brighter in the 0.05 m and 0.025 m treated material.

In each case, after extraction, oxygen was admitted and the solution shaken, yet in no case did light appear either in the undissolved residue or in the solution. The 0.02 m HCl extract was also neutralized, as it

is well known that the acid prevents light-production. As McDermott (23) has simultaneously failed to extract a photogenic substance with oxygen-free aqueous solvents, we may safely conclude that something connected with light-production undergoes decomposition on standing. We shall see later what this substance is.

In 1885 Dubois (9) showed that the elaterid beetle *Pyrophorus noctilucus* contained the substances luciferin and luciferase which are described on page 176. I find that the lampyrid beetles *Photinus*, *Photuris*, and *Luciola* also contain similar substances, but their properties agree with the related bodies previously described in *Cypridina* rather than those described by Dubois for *Pholas* (10), and consequently I have likewise called them photogenin (=luciferase) and photophelein (=luciferin). For instance, firefly photophelein can not be oxidized with light-production by oxidizing agents and is found in many non-luminous forms, the opposite of the condition said to hold for *Pholas* luciferin (=photophelein).

Like *Cypridina* photogenin, firefly photogenin is prepared by allowing an aqueous extract of the luminous organ to stand until the light disappears, and the photophelein is prepared by extracting the firefly with boiling water. Light appears on mixing the two substances. As we shall see, firefly photophelein alone in solution is very stabile, but, together with unboiled extract of luminous or non-luminous parts of the firefly, it is very unstabile. Therefore the photophelein is probably the substance which underwent decomposition when dried powdered luminous tissue was allowed to stand in contact with oxygen-free water for one hour in the experiments described above.

DISTRIBUTION OF PHOTOGENIN AND PHOTOPHELEIN IN THE FIREFLY AND OTHER ORGANISMS.

While photogenin is found only in the luminous gland-cells of the firefly, photophelein is distributed throughout the body of the firefly. An extract of the non-luminous parts of the firefly will give light with photogenin, but only if tested immediately the extract is made. An extract 10 minutes old is found to contain no photophelein unless it has been previously boiled. After boiling the photophelein can be kept for many days without decomposition. There appears to be something in the extract of non-luminous parts of the firefly which causes the photophelein to disappear unless the former is destroyed by heat. That this is the case can be shown by adding unboiled extract of the non-luminous parts to photophelein, when the latter disappears from solution. Because of failure to boil the non-luminous firefly extract, I had for a long time overlooked the existence of photophelein in regions other than the luminous gland. Lack of oxygen retards but probably does not prevent the disappearance of photophelein from non-luminous parts. Note that just as photophelein disappears in contact with photogenin and oxygen with light-production, so it also

disappears in contact with this substance in non-luminous parts, but without light-production. In this respect the firefly agrees perfectly with *Cypridina*.

Among non-luminous forms we find some whose extract will give light with photogenin, whether boiled or unboiled; others whose extract will give no light, whether boiled or unboiled. The first mentioned presumably contain photophelein not readily destroyed by standing, the second are similar to the firefly, and the third may contain no photophelein or some substance very quickly destructive to photophelein or an excess of acid or perhaps merely unstabile photophelein. The exact reasons have not yet been worked out. The following extracts were tried:

Luciola photogenin × caterpillar (sp?) blood	Bright.
Luciola photogenin × caterpillar (sp?) blood, boiled	Bright.
Luciola photogenin × beetle (*Coccinella 7-punctata*) extract	Negative.
Luciola photogenin × beetle (*Coccinella 7-punctata*) extract, boiled	Bright.
Luciola photogenin × beetle (*Glysiphana jucunda*) extract	Negative.
Luciola photogenin × beetle (*Glysiphana jucunda*) extract, boiled	Bright.
Luciola photogenin × beetle (*Anomala rufscuprea*) extract	Negative.
Luciola photogenin × beetle (*Anomala rufscuprea*) extract, boiled	Negative.
Luciola photogenin × grasshopper (*Pachytylus danicus*) extract	Very faint.
Luciola photogenin × grasshopper (*Pachytylus danicus*) extract, boiled	Faint.
Luciola photogenin × myriapod (*Scolopendra* sp?) extract	Faint.
Luciola photogenin × myriapod (*Scolopendra* sp?) extract, boiled	Faint.
Luciola photogenin × ox-blood extract (boiled or unboiled)	Negative.
Luciola photogenin × neutral potato-juice (boiled or unboiled)	Negative.

Note that the oxidases of blood or potato-juice will give no light with photogenin and that they also will give no light with photophelein, even if we add H_2O_2. As we shall see later, although the oxidases can oxidize pyrogallol with light-production, they have nothing to do with light-production by animals (see p. 232).

As we have already seen (p. 182), *Cypridina* photophelein, if concentrated, will give a very faint light with firefly photogenin, and *vice versa*. Firefly photogenin and luminous bacteria prepared in the proper way (p. 214) or *Watasenia scintillans* photophelein (p. 223) also give a faint light, but the converse experiment fails to produce light. *Cavernularia* photophelein gives no light with firefly photogenin, but the converse experiment (p. 205) does. At most the light produced in any of these cross-experiments between distantly related species is very faint and again shows that photogenin and photophelein from different luminous forms possess a certain degree of specificity. As was to be expected the different combination mixtures of these substances in various genera of the Lampyridæ all give light as well as the reciprocal combinations between Lampyridæ and Elateridæ (*Pyrophorus*). Indeed, it may be found that the photogenins from different forms exhibit differences in light-giving power, depending on relationship, similar to the differences in the hemoglobins, or similar to the specificity of the precipitin reactions of different animals.

Photophelein, on the other hand, will not give light with extracts of any non-luminous forms or with any chemical substances.

CONCENTRATION OF PHOTOGENIN AND PHOTOPHELEIN NECESSARY TO PRODUCE LIGHT.

The amount of photogenin in the firefly is greater than the amount of photophelein necessary to combine with it; otherwise we would not be able to obtain photogenin, because it would be completely used up in combination with photophelein.[1] In some luminous animals (*Noctiluca, Cavernularia, Watasenia*) I have utterly failed to demonstrate a photogenin-photophelein reaction under conditions which should be favorable and after many attempts to demonstrate it. The explanation of this result may lie in the presence of equivalent amounts of the two substances.

Both photogenin and photophelein are found in much smaller concentration in the firefly than in *Cypridina*. The photogenin from one firefly (*Luciola parva*), whose average size is 8.2 by 3.1 mm., with luminous organ in the male 2.2 by 1.5 mm. and in the female 2.3 by 0.5 mm., will give just visible light in 1.6 c.c. water, and the photophelein from 1 firefly in 3.2 c.c. water. The greater possible dilution of the photophelein is due no doubt to the fact that the photophelein comes from the whole body, while the photogenin is derived only from the luminous gland. When we compare this with the crustacean *Cypridina*, which will give visible light in 25,600 c.c. or one part of luminous gland in 1,700,000,000 parts of water, we see that the firefly, despite the brilliancy of its light, is not a very powerful light-producer.

PROPERTIES OF PHOTOGENIN AND PHOTOPHELEIN.

As to the chemical nature of photogenin and photophelein, nothing definite is known, except that it is not a fat or fat-like substance. The photophelein is much the more stable substance and can be preserved, until attacked by bacteria, or with chloroform for over 6 months. It dialyzes readily through collodion, is not adsorbed by lampblack, and is not readily affected by ether and benzol. Photogenin, on the other hand, disappears in less than 5 hours at 25° C., is quickly destroyed by ether, benzol, and chloroform, and will not dialyze readily if at all.

In this ready destruction of photogenin by the fat-solvent anesthetics, the firefly resembles *Pholas dactylus* and differs markedly from *Cypridina*. One sample of *Cypridina* photogenin was preserved under chloroform for over 54 days and still gave light with photophelein.

Luciola photogenin is destroyed at about 42°, while the photophelein is still active after 10 minutes' boiling. A bright light is produced on mixing the two at 0° C. Exact destruction temperatures are difficult to determine, however, and Lund (3) finds that in *Photuris pennsylvanica*, if the intact luminous tissues are studied, the light disappears

[1] I have not actually made the experiment to show that photogenin is used up in giving light with photophelein, but it seems highly probable that this is the case in the firefly, as it is in *Cypridina*.

TABLE 10.—*Comparative properties of photogenin and photophelein in Cypridina, firefly, and Pholas.*

Property.	Cypridina.		Firefly.		Pholas (Dubois, **10**).	
	Photophelein.	Photogenin.	Photophelein.	Photogenin.	Luciferin (photophelein).	Luciferase (photogenin).
Destruction temperature.	Boiling 1 to 5 minutes.	70 degrees or above (light disappears 52 to 56 degrees; returns on cooling).	Not destroyed by 10 minutes' boiling.	42 degrees.....	Above 70 degrees....	60 degrees.
Distribution.....	Throughout *Cypridina* and many non-luminous forms.	Luminous organs of *Cypridina*.	Throughout firefly and many non-luminous forms.	Luminous organs of firefiy.	Luminous organs of *Pholas*.	*Pholas* and many non-luminous forms.
Stability.....	Less stabile.....	More stabile.....	More stabile....	Less stabile.....	More stabile	Less stabile.
Porcelain filtration...	Positive.....	Positive.....	Positive.....	.....	With difficulty.....	With difficulty.
Dialysis.....	Easily.....	Very slowly.....	Easily.....	Negative (?)....	Slow.....	Negative.
Adsorption by bone-black and $Fe(OH)_3$.	Positive.....	Positive.....	Negative.			
Light with oxidizing agents.	Negative.....	Negative.....	Negative.....	Negative.....	Positive.....	Negative.
Light with saponin, thymol, NaCl, etc.	Negative.....	Positive.....	Negative.....	Negative.		
Ether solubility.....	Negative.....	Negative.....	Negative.....	Negative.....	Negative.....	Negative.
Concentration just giving light.	1 to 1,700,000,000...	1 to 1,700,000,000.				
Substance from one animal gives just visible light in—	25,600 c.c.....	25,600 c.c.....	3.2 c.c. for *Luciola parva*.	1.6 c.c. for *Luciola parva*.		
Effect of chloroform, ether, benzol, etc.	Fairly stabile.....	Stabile.....	Stabile.....	Quickly affected.	Very slowly affected.	Slowly affected.
Effect of alkalies.....	Not destroyed by n/30 NaOH in 1 hour.	Destroyed by n/125 NaOH in 1 hour.	.....	.....	Forms insoluble alkali-albumin with NH_4OH.	
	Reversible extinction of light by n/125 NaOH if neutralized immediately.					
Effect of acids.....	Destroyed by n/1000 HCl in 1 hour.	Destroyed by n/2000 HCl in 1 hour.	.....	.....	Not precipitated by acetic and H_2CO_3 except in presence of neutral salts.	
	Reversible extinction of light by n/2000 HCl if neutralized immediately.					

Effect of alcohols and acetone.	Reversible extinction of light with 20 per cent alcohol, 16 per cent acetone, and saturated butyl alcohol.	.....	.....	Precipitated by alcohol at 82° unchanged.	
Effect of tannic, picric and phosphotungstic acids.	Reversible extinction of light in n/500 to n/5000 concentration.	.....	.....	Precipitated by picric acid unchanged.	
Effect of KCN	No effect	No effect			
Effect of NaF	.....	.....	.....	.....	Not destroyed by 1 to 100.
Effect of enzymes	.....	.....	.....	.....	Destroyed by trypsin.
Saturation with $(NH_4)_2SO_4$, NaCl, $MgSO_4$, sugar, and glycerine.	Reversible extinction except in case of acid $(NH_4)_2SO_4$.	.....	.....	Precipitated by $(NH_4)_2SO_4$; not by NaCl or $MgSO_4$; insoluble in glycerine.	Suspends activity and returns on dilution.
Color of light	Bluish white	Yellowish white		Greenish blue	

between 45° and 54° and returns on cooling. The maximum temperature from which the light revived was 84° and it usually returned about 50°. These effects are similar to those observed with *Cypridina*.

Very weak concentrations of acids prevent the production of light of the firefly, less weak concentrations of alkalies are necessary, and KCN does not affect light-production in strong (m/100) concentrations.

Table 10 summarizes the characters of photogenin and photophelein (luciferase and luciferin) as found by Dubois for *Pholas* and by myself for *Cypridina* and the firefly. Where a statement runs through both photogenin and photophelein columns it applies to a mixture of the two substances. A blank indicates that the experiment has not been tried.

STUDIES ON A PENNATULID, CAVERNULARIA HABERI.

GENERAL CHARACTERS OF THE ANIMAL AND OF THE PHOTOGENIC MATERIAL.

Panceri (24), working with *Pennatula phosphorea*, has given us the best account of phosphorescence among the pennatulids. He describes the light as coming from eight bands of cells on the outer wall of the stomach and continued into buccal papillæ. They contain a luminous "fatty" matter which can easily be squeezed out as a sort of slime and which does not decompose readily. He also found that on stimulation a wave of light would pass over the colony in any direction at a rate of about 5 cm. per second. In a form like *Cavernularia* he describes also somewhat similar conditions (24, p. 40), but his descriptions are very meager. I have found the Japanese species of *Cavernularia* more favorable for light experimentation than *Pennatula*, and the following pages contain confirmation of Panceri's experiments on the light-waves passing over the colony and a general account of the chemistry of light-production.

Cavernularia haberi is especially abundant in the fjord of Aburatsubo, Japan, near Misaki, the marine station of the Imperial University of Tokyo. The colony of animals lies hidden in the sand and contracts during the day, but at night takes up water and expands, large ones to the length of 2 feet. If stimulated by touching or electrically, or by the addition of ammonia, a slime is formed similar to that produced by most of the *Cnidaria* upon irritation, but differing in that it is brightly luminous. The whole of the outer surface of the colony can form the luminous slime, but not the spongy inner material. The stalk, containing no polyps, is especially brilliant.

The slime may be dried over $CaCl_2$ and will give light when moistened with sea-water or fresh water.

By squeezing *Cavernularia*, from which most of the sea-water has been gently pressed, one can easily obtain a luminous juice which is still luminous when filtered through filter paper and retains its luminescence for several hours. Examination of the filtrate under the microscope in the dark shows that the light comes from minute points of

light which make the field of view look like the starry heavens. If water is added to this sea-water juice, the light is greatly increased, due to the appearance of numerous additional points of light. These points of light come from minute granules and globules easily visible in the filtered juice under the microscope. The addition of water to a dark *Cavernularia* juice which has stood for two days will cause the appearance of light due to the dissolving of the granules and globules. So sensitive are they to the addition of water that 1 drop of fresh-water added to 5 c.c. of juice will produce light, and so bright is the light that the addition of 1 drop of *Cavernularia* juice to 5 c.c. of fresh-water produces a light easily visible when the drop is mixed with the 5 c.c.

By centrifuging the filtered juice, the granules may be partially thrown down and will give a brilliant light if fresh-water is added, while the liquid itself (turbid in appearance) gives a much fainter light. No light is produced upon the addition of water to a parchment-paper sea-water dialysate of the juice, so that light-production is undoubtedly connected with the visible globules and granules of the juice. That it is connected with the solution of the granules is indicated by the fact that fresh-water but not salt-water or isotonic cane-sugar is able to call forth the production of light. The process appears to be similar to the cytolysis of cells, as can be observed by an inspection of table 8, which shows the effect of adding various substances to the dark juice of *Cavernularia*.

The light-giving granules of *Cavernularia* will pass through an alundum filter crucible (R A 84) of the finest pores, but not through a Pasteur-Chamberland filter-tube. The liquid passing through the latter is perfectly clear and non-luminous and gives no light when water is added.

There is no adsorption of the light-producing substance by bone-black or $Fe(OH)_3$.

ELECTRICAL STIMULATION.

The juice of *Cavernularia* filtered through filter paper does not respond to the strongest interrupted induced shocks. The living colony, however, responds readily.

When a galvanic current is passed through one of the excised polyps mounted between non-polarizable electrodes, a flash of light occurs on the make and a series of flashes while the current is passing, which cease on the break. There is no flash of light on the break. A similar response can be observed with *Noctiluca* (2). It will be remembered that Romanes (25) observed a series of contractions in the bell of a medusa during the passage of a galvanic current, and the sartorius muscle of the frog often contracts on the make of a galvanic current, remains contracted during the passage of the current, and relaxes on the break.

If the whole colony be stimulated by weak induced shocks, there is a local production of light. There is usually no response to a single shock, but a ready response to three or more sent in in rapid succession.

With stronger stimuli, a wave of light, easily followed by the eye, passes over the colony in each direction from the point stimulated. With interrupted induced shocks, a series of waves of light follow one another in quick succession (not corresponding to the number of stimuli, however), reminding one of the series of electric shocks given out by the torpedo, only on a slower scale. The time-interval between separate flashes no doubt corresponds to the refractory period of the cells concerned.

On pressing deeply into the tissue and stimulating strongly, a much brighter light-response also results, which very slowly moves away from the point of stimulation and usually does not extend more than 2 or 3 cm. At the same time the whole colony contracts, the polyps are drawn in, and in this condition do not respond to electrical stimulation by light-production.

The wave of light above mentioned will pass in any direction over the colony and across a cut around the middle of the colony involving the whole of the external tissue. Some inner tissue must therefore be capable of conducting the stimulus.

LACK OF OXYGEN.

That the production of light by *Cavernularia* is an oxidation can be very readily determined by passing a current of hydrogen through the juice, when the light disappears, but promptly reappears upon admitting oxygen. A sample of phosphorescent juice kept 24 hours in an atmosphere of hydrogen gave light when air was admitted.

Considerable oxygen is necessary, as may be seen by keeping the juice in a tall test-tube, when it lights only at the surface in contact with air. The light appears through the tube, however, if mixed with air.

REDUCTASE, OXIDASE, AND CATALASE.

A tube of *Cavernularia* juice shut off from the air very quickly reduces (decolorizes) methylene blue. The blue color reappears when oxygen is admitted. This reaction has nothing to do with light-production, however, as it is given also by a non-luminous sea anemone (*Anthopleura xanthogrammica*) and many other animal tissues which can not produce light.

Unboiled *Cavernularia* juice also gives oxidase reactions upon addition of H_2O_2 with guaiac, pyrogallol (slight), *a*-napthol, para-phenylen diamine, indo-phenol reagent, ortol, and pyrocatechin. The boiled juice gave a slight positive test with guaiac, para-phenylen diamine and the indo-phenol reagent. Many other non-luminous tissues give these reactions, however, and there is abundant evidence to show that

light-production in animals is not connected with the ordinary oxidases (peroxidases) (see p. 232).

As in all other organic tissues, catalase is also present in the juice of *Cavernularia*.

TEMPERATURE.

The juice of *Cavernularia* still gives a good light at 0° C., as do pieces of the colony if stimulated.

On raising the temperature, the light of *Cavernularia* juice disappears at 52° and does not reappear on cooling. Pieces of the colony slowly heated begin to light spontaneously at about 40°.

The light of *Noctiluca* (2) disappears at 48°, *Cypridina* at about 54°, a hydroid (*Sertularia* sp.) at 54°, the firefly (*Luciola*) at 42°, and luminous bacteria at 38°.

PHOTOGENIN AND PHOTOPHELEIN.

Unlike the firefly, *Cypridina*, and *Pholas dactylus*, it is impossible to separate the luminous juice of *Cavernularia* into two substances, photogenin and photophelein (luciferase and luciferin), one destroyed by boiling, the other not, which will give light when mixed. We can not, for instance, cause light to appear in *Cavernularia* juice which has stood until the light has disappeared by adding fresh luminous *Cavernularia* juice heated to boiling and then cooled; neither can we obtain light by adding juice heated to temperatures below boiling (88°, 81°, 71°, 61°, or 52° C.) and then cooled.

Another species of pennatulid, *Pennatula* sp., as also *Noctiluca* and the squid *Watasenia scintillans*, behaves as does *Cavernularia*. None of these organisms gives the photophelein-photogenin (luciferin-luciferase) reaction, for reasons at which we can only guess. The photogenin or photophelein may be, either of them, very unstabile, or there may be sufficient photophelein to use up all the photogenin. The evidence in this case seems to indicate that the photophelein is unstabile, as we can obtain a faint light with *Cypridina* photophelein or firefly photophelein (both prepared with sea-water) and the non-luminous *Cavernularia* juice (photogenin). *Pennatula* and *Noctiluca* photophelein gave negative results with *Cavernularia* juice, and *vice versa*.

TABLE 11.—*Effect of standing on light-producing power of Cavernularia juice.*

Juice + water	Bright light.
Juice 12 hours old at 20° C. + water	Bright light.
Juice 24 hours old at 20° C. + water	Fair light.
Juice 48 hours old at 20° C. + water	Faint light.
Juice 72 hours old at 20° C. + water	None.

The question may be asked, what substances are able to cause light to appear in the juice of *Cavernularia* which has stood until completely dark? Usually 4 to 6 hours are sufficient time for the juice to lose its luminescence. It is still capable of giving out a bright light if we add fresh-water (but not sea-water) to it, and it retains this potentiality

for over two days at 20° C. (see table 11) and a shorter time at higher temperatures. As only fresh water and not sea-water will call forth the light, and as we know that light-production is connected with the granules of the juice, the process appears similar to the cytolysis of cells—*i. e.*, to the swelling and solution in fresh water of the granules which the cells contain. By this means something is liberated from the granules of *Cavernularia* juice which oxidizes with light-production. Table 12 gives the results of adding various other substances, pure or dissolved in sea-water, to the dark *Cavernularia* juice.

TABLE 12.—*Effect of substances in calling forth light from non-luminous Cavernularia juice.*

Solution or substance added.	Character of light.	Solution or substance added.	Character of light.
50 per cent sea-water	Bright light.	Ortol crystals	Faint light.
66 per cent sea-water	Fair light.	Hydrochinon crystals	None.
75 per cent sea-water	Fainter light.	Resorcin crystals	None.
80 per cent sea-water	Very faint light.	Pyrocatechin crystals	None.
90 per cent sea-water	None.	Pyrogallol crystals	None.
Water	Bright light.	Ba_2O_2 powder	None.
Sea-water	None.	Na_2O_2 powder	None.
Sea-water evaporated to one-half volume	None.	$KMnO_4$ crystals	None.
m cane-sugar	None.	$K_2Cr_2O_7$ crystals	None.
m/2 cane-sugar	Faint light.	$FeCl_3$	None.
m/4 cane-sugar	Fair light.	$FeSO_4$ crystals	None.
m/8 cane-sugar	Good light.	$K_4Fe(CN)_6$	Faint.
Chloroform-saturated sea-water	Faint light.	*Laonome japonica* (an annelid) blood	None.
One-half chloroform-saturated sea-water	Very faint light.	Dried ox-blood extract in sea-water	None.
One-quarter chloroform-saturated sea-water	None.	*Lepas anatifera* extract	None.
Chloretone-saturated sea-water	None.	*Lepas anatifera* extract, boiled	None.
One-half chloretone-saturated sea-water	None.	*Chiton* sp. extract	None.
One-quarter chloretone-saturated sea-water	None.	*Chiton* sp. extract boiled	None.
Thymol-saturated sea-water	Fair light.	*Onchidium* sp. extract (a pulmonate mollusk)	None.
One-half thymol-saturated sea-water	Faint light.	*Onchidium* sp. extract, boiled	None.
One-quarter thymol-saturated sea-water	Very faint light.	*Dolabella* sp. blood (a nudibranch)	Faint.
Benzol	Faint light.	*Dolabella* sp. blood, boiled	None.
Ether	None.	*Sepia esculenta* blood	None.
Chloroform	None.	*Sepia esculenta* blood, boiled	None.
Chloretone crystals	Very faint light.	*Panulirus japonica* blood	Very faint light.
Thymol crystals	Faint light.	*Panulirus japonica* blood, boiled	None.
Chloral-hydrate crystals	Faint light.	*Cypridina hilgendorfii* photophelein in sea-water	Fair light.
Saponin powder	Fair light.	*Luciola vitticollis* photophelein in sea-water	Fair light.
Oleic acid	Faint light.		

Note from table 12 that light-production is not due to dilution of the salts of sea-water by adding fresh-water, since m cane-sugar does not call forth the light. Note also that many cytolytic substances (chloroform, benzol, thymol, etc.) give light, but not the oxidizing

agents, Na_2O_2, $KMnO_4$, etc. The blood of certain invertebrates also causes very faint light-production, but we can not be sure that this is not due to the fact that the blood is somewhat less concentrated than sea-water, although the determinations of other closely allied forms show the salt-content to be the same as the sea-water in which they live.

CHEMICAL REACTIONS.

The luminous juice of *Cavernularia* contains the luminous substance, but mixed with many substances, so that mere chemical tests on the juice are of no value in determining the chemical nature of the luminous substance. The luminous material is salted out along with the other proteins, but does not retain its power to phosphoresce long enough to be manipulated by the ordinary chemical methods. The following paragraphs give the results of precipitation by $(NH_4)_2SO_4$, picric acid, alcohol, etc.

If to the fresh filtered luminous juice of *Cavernularia* we add sugar, NaCl, $MgSO_4$, $(NH_4)_2SO_4$ to saturation, or 5 volumes of glycerine, the light disappears. A heavy precipitate forms in $(NH_4)_2SO_4$, a small precipitate in $MgSO_4$ and practically none in NaCl. The precipitates are soluble in sea-water. No precipitate is formed in sugar or glycerine. If poured into fresh-water or sea-water immediately after the light has disappeared, light is produced by all solutions. If the tubes are allowed to stand for a day, no light is produced by any tube on pouring into fresh-water or sea-water.

If alcohol or acetone (3 vols.) is added to the juice, a precipitate forms and the light disappears. The precipitate is insoluble and gives no light in sea-water or fresh-water.

The alkaloidal reagents—picric acid, phosphotungstic acid, and tannic acid—immediately cause precipitation with disappearance of light. The precipitates are insoluble in sea-water or fresh-water and produce no light.

Normal acetic acid or n NH_4OH, added drop by drop, produce no precipitate, although the light disappears in weak concentrations.

ANESTHETICS.

Ether, chloroform, and benzol cause a fairly rapid disappearance of the light in the order named, the ether most rapid. In turpentine, chloral hydrate, thymol, and chloretone the light stays for a long time and then disappears. There is no reappearance of light from any of the tubes if poured into fresh-water or sea-water unless we do so immediately the light has disappeared, and then the light is very faint and momentary.

In other words, the juice can not be anesthetized. Even in concentrations of alcohol, ether, chloroform, or butyl alcohol, which cause a slow disappearance of light, there is no recovery or only a momentary

faint glow upon dilution of the solutions with sea-water. The alcohols give similar results. About 16 per cent ethyl alcohol will cause the light to disappear in one minute, but there is no recovery or only a very faint momentary glow on diluting with sea-water. Saturation with butyl alcohol gives a similar result.

Since reversibility is the *sine qua non* of anesthesia, we must conclude that this juice can not be anesthetized, in this respect differing markedly from the granule-free *Cypridina* luminous secretion which can be anesthetized by the alcohols. Also many luminous organisms—for instance, luminous bacteria—can be truly anesthetized (see p. 219).

POTASSIUM CYANIDE.

Potassium cyanide has no inhibiting effect upon the light-production of *Cavernularia* juice. Even in m/40 concentration the light is still bright after 90 minutes, a result agreeing with all other experiments on luminous organisms (bacteria, firefly, *Cypridina*, *Noctiluca*).

SUMMARY OF RESULTS FROM CAVERNULARIA.

1. The light of *Cavernularia* comes from a luminous slime, a secretion of gland-cells over the outer surface of the colony.

2. The secretion contains small granules which can be seen to emit the light. On standing the light from a sea-water extract of *Cavernularia* slowly disappears and will not return on shaking, but reappears if fresh water is added to the juice.

3. The light substance—*i. e.*, the granules—will pass an alundum filter (R A 84) but not a Chamberland filter. It is not adsorbed by boneblack or $Fe(OH)_3$ and will not dialyze.

4. The light-producing granules do not respond to electrical stimulation, but the colony gives light with galvanic or induced currents.

5. With galvanic currents a flash appears on the make and a series of flashes while the current is passing, which cease on the break.

6. Upon stronger stimulation (with induced currents) a wave of light passes over the colony in all directions from the point stimulated. This wave will pass through the deeper tissues when all the ectodermal tissues are cut.

7. A considerable amount of oxygen is used up in light-production and no light appears in its absence.

8. *Cavernularia* juice will decolorize (reduce) methylene blue in absence of oxygen, and it also contains peroxidases and catalase.

9. The light is still bright at 0° C. Fragments of *Cavernularia* give off light spontaneously at 40° C., which disappears at 52° C. and does not return on cooling.

10. The photogenin-photophelein reaction is not given by *Cavernularia*, but a faint light can be obtained with a non-luminous *Cavernularia* juice (photogenin) and *Cypridina* or firefly photophelein.

11. The production of light by the granules appears similar to the cytolysis of cells, as it occurs with water (but not isotonic cane-sugar) and certain cytolytic substances (saponin, chloroform, benzol, oleic acid).

12. The light-producing substances are salted out along with the proteins, but are not stabile enough for chemical manipulation.

13. The light-producing granules can not be anesthetized.

14. Potassium cyanide has no effect on light-production.

STUDIES ON LUMINOUS BACTERIA.

DESICCATION AND EXTRACTION WITH FAT SOLVENTS.

Luminous animals may be divided into two classes—those in which the luminous material is burnt within the living cell (firefly, fungi) and those in which it is secreted by the cell outside (many worms, crustacea, and myriapods). As first shown by Molish (8) the luminous substance must be burnt within the bacterial cell, since a dense emulsion of the luminous bacteria may be separated from the medium by a Chamberland or Berkefeld filter and a clear dark filtrate with no trace of phosphorescence obtained. I have repeated Molish's experiment and can confirm him. An alundum filter crucible was used. Can this photogenic substance in the cell be freed of other cell material and obtained in a more or less pure state? We know that the bacteria can be dried, and when moistened again will phosphoresce, even though the majority are not living, and will give rise to no new growth if inoculated in a suitable culture medium.[1]

These dried bacteria form the material used for extraction purposes. The organisms are best grown in bulk in a thin layer of peptone (1 per cent), glycerine (1 per cent), sea-water nutrient fluid covering the bottom of a white enameled pie-plate and covered by another pie-plate, the whole readily sterilized and serving as a large Petrie dish. The medium must be faintly alkaline to phenolphthalein. The bacteria are easily collected by centrifuging. The dense mass of centrifuged bacteria is then spread in a thin layer on filter paper or on glass wool, placed in a desiccator, and dried over $CaCl_2$ in a vacuum. Spreading on glass wool has the advantage that the glass wool may be ground up in a mortar and a powder obtained which phosphoresces when moistened, but the powder does not give so brilliant a light as does the filter paper containing dried bacteria. It is well to wash the glass wool with several changes of water to remove alkali. Such strips of dried bacteria-impregnated filter paper can be extracted with boiling

[1] I have on two occasions obtained no luminous growths from bacteria which had been dried on filter paper and afterwards moistened (light was produced) with sterile sea-water and placed on nutrient agar culture medium. In the majority of cases, however, colonies of brilliantly luminous bacteria result. These colonies are relatively few in number, indicating that *most* of the bacteria are killed by drying.

ether or cold absolute alcohol for 12 hours without losing their power to phosphoresce when the solvent has been removed and they are again moistened. Colonies of luminous bacteria sometimes appear when the filter paper is placed on a nutrient medium, even after such rigorous treatment with ether and alcohol and other fat solvents. Büchner and Gaunt (26) obtained similar results with the acetic-acid-forming bacteria of beer (*Mycoderma aceti*). The dried acetic bacteria are not all killed by extraction with acetone; moist bacteria are invariably killed.[1]

In testing the solubility in fat solvents, strips of filter paper containing the dried bacteria were placed in the solvents in sterile tubes for a definite time at a definite temperature, the solvent completely removed, and the filter paper tested for light-production by adding sterile sea-water. A plus mark (+) indicates light, a minus mark (−) indicates no light. Controls, untreated with any solvent, always gave a good light. Table 13 gives the results. In the last column are similar results obtained with the dried powdered luminous organs of the firefly. The amyl alcohol and ethyl butyrate were completely removed by washing with ether.

The great majority of fat solvents extract nothing which is essential to light-production, as can be seen from the table. Chloroform might have extracted something, as the material glows only faintly after chloroform treatment. I have, however, evaporated the chloroform extract to dryness *in vacuo* and added water as well as a water extract of luminous bacteria (in itself non-luminous; possibly containing a second necessary substance) to the residue without obtaining light-production. The same result was obtained with the residue of the boiling alcohol extract, so that we must conclude that the chloroform and boiling alcohol extract nothing, but rather destroy the photogenic material. The temperature of boiling alcohol, 78.4°, is not destructive to the photogen. These results are very similar to my previous results on firefly material, as may be seen by inspecting the last column. The photogen of the firefly is not weakened by chloroform or acetone or a boiling mixture of equal parts alcohol and ether, but does suffer from carbon disulphide. Otherwise the results are the same.

The strips of filter paper moistened with sterile sea-water were then transferred under sterile conditions to nutrient agar to see if colonies of luminous bacteria would result. It was found that not in every case, but in at least one experiment out of several tried, luminous colonies were obtained after extraction of the material with ether, alcohol, toluol, acetone, and benzol.

I have already mentioned the fact that dried bacteria will glow if moistened after extraction with cold (20°) absolute alcohol, and also the

[1]I find that extraction of dried luminous bacteria with 95, 80, 50, or 35 per cent alcohol kills them all and no new growths appear. Also if the moist bacteria are treated with a large excess (50 volumes) of absolute alcohol or acetone for 10 minutes and then rapidly dried no growth is possible. Bacteria so treated have also lost their power to phosphoresce when moistened.

fact that if 50 volumes of absolute alcohol are added to a mass of moist centrifuged bacteria and they are then shaken for 10 minutes, the alcohol removed, and bacteria quickly dried, no phosphorescence is obtained on moistening this dry powder. Neither will dried bacteria phosphoresce if extracted with 95, 80, 70, 50, or 37 per cent alcohol. What is the explanation of this? The alcohol does not dissolve out a luminous substance. Water appears to exert some influence. Perhaps we may explain the above result as merely an example of the effect of alcohol on dry albumins as opposed to albumin solutions. Powdered egg

TABLE 13.

Solvent.	Temperature (in degrees C.).	Time of extraction (in hours).	Dried bacteria.	Dried firefly.
Ether (cold)	20	24	+	+
Ether (hot)	35	24	+	+
Chloroform (cold)	20	12	+	+
Chloroform (hot)	61	12	+ faint.	+
Ethyl alcohol (cold)	20	12	+	+
Ethyl alcohol (hot)	78.4	12	−	−
Alcohol and ether (equal parts, boiling).	46	12	− to faint light.	+
Acetone (cold)	20	12	− to faint light.	+
Acetone (hot)	56.3	12	−	+
Carbon tetrachloride	20	24	+	+
Carbon bisulphide	20	24	+ fair, light.	−
Toluol	20	12	+	+
Toluol (hot)	100	12	−	−
Benzol	20	12	+ fair.	Not tried.
Benzine (petroleum ether)	20	24	+ fair.	+
Amyl alcohol	20	24	+	+ faint.
Ethyl butyrate	20	24	−	−
95 per cent alcohol	20	24	−	+very faint.
80 per cent alcohol	20	24	−	−
70 per cent alcohol	20	24	−	−
50 per cent alcohol	20	24	−	−
35 per cent alcohol	20	24	−	−
Material kept dry at	78.4	12	+ fair.	+
Material kept dry at	100	12	−	−

albumin can be extracted with absolute alcohol (or acetone) for 6 hours and is still readily soluble in water. But if a concentrated aqueous solution of albumen is precipitated by a large excess of alcohol (or acetone), even though the alcohol (or acetone) be removed within 15 minutes, the precipitate of albumen is found to be practically insoluble in water. It would be futile to discuss the matter without further experimental results, but the results seem to indicate that in photogenesis there is involved a substance which in the moist state is irreversibly precipitated (or changed) by alcohol and that it is probably protein in nature.

At any rate, the experiments show (1) that the photogenic material is not a fat or a fatlike body soluble in fat solvents, and (2) that phosphorescence does not depend on the living cell, since many of the dried

bacteria which can still phosphoresce when moistened will give rise to no new colonies.

The question may now be raised as to whether bacteria whose structure has been completely destroyed by grinding in the dry state will phosphoresce on moistening. Oxidation can not take place so long as water is absent. Experiment shows that they will not phosphoresce, as the following procedure indicates: Luminous bacteria dried on glass wool are powdered in a porcelain mortar and divided into two equal parts, A and B. A was then ground in the porcelain mortar for 20 minutes with pure quartz sand; B was thoroughly mixed in another mortar with an equal volume of sand, previously ground for 20 minutes, and exposed to the air during the time of grinding A. In this way the effect of quartz powder or the possibility of absorbing moisture from the air would be the same for the unground bacteria B or the ground bacteria A. On moistening B with sterile sea-water, a good phosphorescence appeared, while the ground material gave no light with sea-water. I have repeated the experiment with the same result and feel that there are no possible sources of error. Microscopic examination shows the sand to be ground to the size of the bacteria or smaller and it is well known that even the smallest cells may be broken up by grinding with sand. I find that the dried luminous organs of the firefly likewise lose their power to phosphoresce if thoroughly ground with sand. This result differs from that of McDermott (21), who finds that firefly tissue can be frozen and ground in liquid air without losing its power to phosphoresce. Both A and B were inoculated on agar nutrient medium. The ground bacteria, A, gave rise to no luminous colonies, while the unground bacteria, B, did develop several luminous colonies—further proof that the ground bacteria were wholly broken up and destroyed.

EFFECT OF LACK OF OXYGEN.

Many observers have shown that if luminous bacteria are suspended in sea-water and the oxygen removed the light disappears and again returns when oxygen is readmitted. Will the light reappear if the cells are in the meantime cytolyzed? I have made many attempts to obtain extracts of cells broken up in absence of oxygen which would give light if oxygen were readmitted. All these efforts have failed. The result might have been anticipated by the work of MacFadyen (27), who found that luminous bacteria subjected to the action of liquid air did not phosphoresce at that low temperature, but did phosphoresce as soon as warmed again; further, that if the cells were broken up by grinding at the temperature of liquid air, there was no phosphorescence on rewarming. MacFadyen worked, however, in the presence of oxygen and moisture, and we might suppose that a slow oxidation—too slow to produce light—went on in the material broken up at low temperatures with consequent exhaustion of the photogenic material.

In my experiments the moist bacteria have been broken up (cytolyzed) in absence of oxygen by (1) oxygen-free distilled water and (2) toluol. All marine cells can be cytolyzed by distilled water or fat solvents.

In the first method a dense mass of bacteria are placed in a vessel from which the air is exhausted by an air-pump (see apparatus, p. 195). The bacteria stop glowing, but reglow if air is again admitted. Then oxygen-free distilled water is allowed to flow onto the bacterial mass and it is thoroughly shaken. No light appears (indicating that the water is oxygen-free), and 5 to 10 minutes later, if oxygen is added, still no light is emitted. If there is a definite soluble photogenic substance in the bacterial cell it should have passed into solution in the water when the cell was cytolyzed, and, provided no decomposition took place, it should have glowed when oxygen was readmitted. Even if we assume that the cell was not completely cytolyzed, the photogen, if a stable substance, although one unable to pass the cell surface, should have glowed within the cell.

In the second method a dense emulsion of the bacteria in sea-water is rendered non-luminous by removing the oxygen. Then a drop of toluol is added without admitting oxygen (air). The emulsion is shaken and no light appears. In a few minutes air is admitted and still no light appears. Similar experiments with ether, chloroform, and carbon tetrachloride gave similar results. Thus if the cells are broken up the photogen disappears even though it has not been oxidized, for no oxygen was present. The toluol itself does not destroy the photogenic substance, as evidenced by the treatment of dried bacteria with toluol. Luminous bacteria in oxygen-containing sea-water to which a drop of toluol, ether, chloroform, or carbon tetrachloride is added very quickly stop phosphorescing. I explain this as due to the fact that on cytolysis of the cell the oxidation processes run riot and the available store of photogen is rapidly used up. The same explanation may be applied to the loss of light in distilled water. We may compare the conditions in bacteria to the conditions in a potato cell. When the cells of the potato are crushed or when their surface is destroyed by toluol or ether or chloroform, dark melanin oxidation products are rapidly formed, but if the potato is cut and the cut cells well washed to free them of their cell-contents, no blackening occurs, although the lower intact cells at the cut surface are exposed to atmospheric oxygen and only separated from it by their plasma membranes. A destruction of these membranes would immediately cause oxidations within to proceed rapidly.

The conclusion drawn from the above experiments has been confirmed by allowing oxygen-free sea-water to come in contact with dried bacteria in a hydrogen atmosphere. If, after 15 minutes, oxygen is admitted, no glow is observed, although dried bacteria instantly glow for a short time if moistened with oxygenated sea-water.

All the above experiments, then, point to the conclusion that if the cell is broken up while moist or if the dead cells stand in contact with water for any length of time, even though no oxygen be present, nevertheless the photogenic substance undergoes decomposition, a conclusion in harmony with my work and that of McDermott on the firefly. As we have seen, extraction of the dried firefly luminous organs with oxygen-free solvents will give no phosphorescent solutions on admitting oxygen, because of this instability of the photogen (p. 196). On the other hand, the substances in *Cypridina* extract are stable in oxygen-free water (see p. 176).

In the normal living bacterial cell (or firefly cell) I assume the photogen to decompose through oxidation with light-production. If the living bacteria are kept in sea-water from which all oxygen has been removed and they stop glowing, they will still glow strongly if oxygen is readmitted, even after a period of 24 hours. It is therefore obvious that the breaking-up of the photogen in absence of oxygen does not occur in the intact bacteria, but only in those whose normal "structure" has been destroyed by cytolysis. I am inclined to believe that the surface layer of the cell is the "structure" involved.

PHOTOGENIN AND PHOTOPHELEIN.

As we have just seen, the light of a mass of bacteria disappears almost instantly if they are broken up by any of the usual cytolytic agents. This result is no doubt due to the fact that they continually burn their light-producing substances as soon as they manufacture them. The substances are therefore present in very small quantity. For this reason we might expect to fail in demonstrating the existence of photogenin and photophelein, although we must remember that forms like *Cavernularia,* which contain a large amount of luminous material, also fail in showing the photogenin-photophelein reaction.

Since it is practically impossible to grind the luminous bacteria in sea-water and so prepare an extract of photogenin, we are forced to prepare our extract by cytolyzing with distilled water and thus obtain a dark solution containing swollen suspended bacteria. This fluid will give no light, however, on adding a suspension of bacteria heated to boiling. Thinking that bacterial photophelein as well as photogenin might be destroyed by boiling temperature, I tried heating to lower temperatures, 90°, 80°, 70°, 60°, and 50°, for 2 minutes, but in no case did light appear on mixing with photogenin. Neither did these solutions give light on mixing with firefly photogenin, but we can obtain light from a bacterial photophelein prepared in the following way: by adding absolute alcohol to a dense mass of the bacteria, then removing the alcohol by centrifuging, and quickly drying by evaporation *in vacuo.* The resultant powder gives no light with water, but does cause firefly photogenin to phosphoresce very faintly. Similar material

prepared with acetone instead of alcohol gave no light with firefly photogenin. In order to obtain a bacterial powder which will give light with firefly photogenin it is necessary to remove the alcohol and dry as quickly as possible; otherwise the photophelein is destroyed.

Addition of ether to a mass of luminous bacteria causes the light to disappear rapidly and it is possible that the photogenin is destroyed by ether and not the photophelein; but bacteria so treated, after the ether had been evaporated by a current of air, gave no light with firefly photogenin. Negative results were obtained also with an alkaline (m/12 KOH) extract of luminous bacteria neutralized with HCl. A precipitate of nucleoprotein (?) is produced on neutralization. Apparently the bacterial photophelein is formed in such small quantities from its precursor or is so unstable that all methods of obtaining it have failed, with the exception of the rapid precipitation by alcohol.

TABLE 14.

Organism.	Observer.	Minimum.	Maximum.
		° *C.*	° *C.*
Bacterium phosphorescens....	Lehmann...	−12	39.5
Bacterium phosphoreum.....	Molisch....	−5	28
Light bacteria.....	Tarchanoff..	−7	37–50

TEMPERATURE.

The temperatures shown in table 14 have been recorded for the extinction of the light of luminous bacteria (28). The variations in the results suggest that further observations were desirable. Accordingly luminous bacteria, isolated from fish, were grown on absorbent cotton saturated with beef-broth-peptone-glycerol culture medium. Free access of air between the cotton fibers supplies the conditions for a brilliant light and at the same time an excellent means of handling the bacteria. A wisp of cotton strongly phosphorescent with bacteria was placed in a very thin-walled glass tube about 2 mm. in diameter and attached to a thermometer bulb as for melting-point determinations. On slowly raising the temperature, the light (to a dark-accustomed eye) becomes dim at 30°, very dim at 34°, and disappears at 38°. On slowly lowering the temperature the light weakens at 0°, is very dim at −7°, and disappears at −11.5°. These values agree best with those given by Lehmann for *Bacterium phosphorescens* and do not greatly exceed the usual temperature limits of activity of organisms.

Bacteria raised to 38°, and then cooled, phosphoresce only very dimly, but, as first observed by MacFadyen (27), again glow brilliantly at room temperature even after an exposure to liquid air.

OXIDASE AND CATALASE.

Many enzymes are found in bacteria in a condition which defies extraction except by means of the Büchner press, and it is difficult to obtain luminous bacteria in sufficient quantity for this method. The oxidases seem to be contained in these bacteria in this endoenzyme condition. All my efforts to obtain an oxidase in solution which will oxidize the common oxidase reagents—guaiac, *a*-napthol, para-phenylen diamine, phenolphthalin, and pyrogallol—have failed, even though hydrogen peroxide is added.

Similar results have been obtained by other workers. Gessard (29) showed that a melanogenic variety of *Bacterium pyocyaneum* would turn tyrosin brown, but he was unable to separate a solution of tyrosinase from the bacteria. Lehmann and Sano (30) found also that *Actinomyces chromogenes, Bacterium putidum,* and *B. phosphorescens* would oxidize tyrosin in the culture medium, but *Vibrio indicus,* which phosphoresces strongly, *Sarcina lutea, B. typhi, B. coli,* and many others would not. They found in addition that a substance oxidizing aloin and giving a very weak guaiac reaction could be extracted from *Actinomyces chromogenes, B. putidum,* and *B. phosphorescens* by a mixture of two parts glycerine to one of water, but no tyrosinase reaction could be obtained with this extract. Here also the tyrosinase is apparently in an endoenzyme condition.

As we shall see (p. 225) the vegetable oxidases are capable of oxidizing pyrogallol with light-production, but many attempts to produce light with pyrogallol and extracts of luminous bacteria failed.

Catalase also occurs in the luminous bacteria with which I worked, but in small amount.

LUMINOUS BACTERIA AND OSMOTIC PRESSURE.

Most marine animals require an external medium of some definite osmotic pressure, and they will not live below this limit; some marine forms (*Fundulus*) are independent of an external osmotic pressure. Luminous bacteria belong to the first category, as indicated in table 15. They will not live in dilute sea-water, but they will live in it if its osmotic pressure is raised by some inert substance like isotonic cane-sugar. Note also that some salt is necessary for the continued production of light, as the bacteria no longer glow after 24 hours' immersion in m sugar, a fact of no great surprise, as unicellular fresh-water luminous animals are unknown.

In these experiments, as well as those with the salts of sea-water, acid and alkalies, and alcohols, one drop of a dense emulsion of luminous bacteria (a form isolated from squid at Woods Hole, Massachusetts) was added to 30 c.c. of solution in an uncorked Erlenmeyer flask and the whole thoroughly mixed. For comparative observations it is essential that the eye be thoroughly adapted to the dark and that each

flask be oxygenated by shaking, before judging as to the emission or absence of light. Observations were made after 10 minutes, 1 hour, and 24 hours.

TABLE 15.—*Effect of dilution of sea-water with water and with m cane-sugar solution.*

Dilution with water.					Dilution with m cane-sugar.				
Parts sea-water.	Parts water.	Light after—			Parts sea-water.	Parts m sugar.	Light after—		
		10 min.	1 hour.	24 hours.			10min.	1 hour.	24 hours.
2	1	+	+	+	2	1	+	+	+
1	1	Faint.	Faint.	−	1	1	+	+	+
1	2	Faint.	Very faint.	−	1	2	+	+	+
1	4	Faint.	−	−	1	4	+	+	Faint.
1	6	Very faint.	−	−	1	6	+	+	Faint.
1	10	−	−	−	1	10	+	+	Faint.
1	14	−	−	−	1	14	+	+	−
1	20	−	−	−	1	20	+	+	−
Sea-water.....		+	+	+	m cane-sugar...		+	+	−
Water.....		−	−	−					

LUMINOUS BACTERIA AND BALANCED SOLUTIONS.

Not only must luminous bacteria be bathed with a certain concentration of salts, but any salt of sea-water is not sufficient. It must be a particular salt, although luminous bacteria, as indicated in table 16,

TABLE 16.—*Effect of various combinations of the salts of sea-water.*

Salt combinations.	Light after—		
	10 min.	1 hour.	24 hours.
Sea-water	+	+	+
Artificial sea-water, m/2(100 NaCl+2.2 KCl +2 $CaCl_2$+ 10 $MgCl_2$) +n/4000 NaOH	+	+	+
Neutral artificial sea-water	+	+	Faint.
m/2 NaCl	+	+	Faint.
m/2 KCl	+	Faint.	−
m/3 $CaCl_2$	−	−	−
m/3 $MgCl_2$	−	−	−
m/2(100 NaCl+2.2 KCl)	+	+	Faint.
m/2(100 NaCl+2 $CaCl_2$)	+	+	Faint.
m/2(100 NaCl+10 $MgCl_2$)	+	+	Faint.
m/2(100 NaCl+2.2 KCl+2 $CaCl_2$)	+	+	Faint.
m/2(100 NaCl+2.2 KCl+10 $MgCl_2$)	+	+	Faint.
m/2(100 NaCl+2 $CaCl_2$+10 $MgCl_2$)	+	+	Faint.

are not so affected by the pure monovalent cations of sea-water as are most cells.

Note the independence of these bacteria of a balanced medium. The bacteria live and phosphoresce in pure NaCl without the addition

of any bivalent ions. This is true even when the solution is changed three times to remove the last traces of Ca in the solution. KCl is also relatively non-toxic, although more so than NaCl. $CaCl_2$ and $MgCl_2$ are very toxic when alone. All combinations of NaCl with the other ions of sea-water sustain the bacteria well, except that they are neutral media, and hence the phosphorescence is dimmed after 24 hours. That pure NaCl should have so little effect on light-production is astonishing when we consider its poisonous effect on other marine organisms and tissues, particularly on ciliated cells.

TABLE 17.—*Effect of acid and alkali.*

Concentration of acid and alkali added to Mg-free sea-water, m/2(100 NaCl+2.2 KCl+2 $CaCl_2$) in Syracuse watch glasses.	Light after—		
	10 min.	1 hour.	24 hours.
n/2000 HCl	−	−	−
n/4000 HCl	Faint.	−	−
n/8000 HCl	+	Faint.	−
n/16000 HCl	+	+	−
n/32000 HCl	+	+	Faint.
n/500 valerianic acid	−	−	−
n/1000 valerianic acid	Faint.	−	−
n/2000 valerianic acid	Faint.	−	−
n/4000 valerianic acid	+	Faint.	−
n/8000 valerianic acid	+	+	−
n/16000 valerianic acid	+	+	Faint.
n/500 NaOH	−	−	−
n/1000 NaOH	−	−	Faint.
n/2000 NaOH	+	+	+
n/250 methyl amine	−	−	−
n/500 methyl amine	Faint.	−	Faint.
n/1000 methyl amine	Faint.	+	Faint.
n/2000 methyl amine	+	+	+
Mg-free sea-water	+	+	Faint.
Sea-water	+	+	+

EFFECT OF ACIDS AND ALKALIES.

As NaOH precipitates the Mg of an artificial sea-water, it is always necessary to use Mg-free sea-water in order that the concentration of NaOH may not be reduced. The acids were also added to a neutral artificial Mg-free sea-water as well as the alkalies, in order to obtain comparative results. Table 17 gives the results. The return of light in n/1000 NaOH and n/500 methyl amine after 24 hours was possibly due to neutralization of alkali through absorption of CO_2.

As was to be expected, acids and alkalies were found to prevent light emission in very weak concentration, the acids in much weaker concentration than the alkalies. In fact, the bacteria are very sensitive to acid and will not even phosphoresce with any brilliancy in a neutral medium.

The organic acid (valerianic) and alkali (methyl amine) have less effect than the inorganic, a result at variance with my results for other organisms which are usually affected more readily by the weak than by the strong acids and alkalies (**12** and **13**).

EFFECT OF HOMOLOGOUS ALCOHOLS (ANESTHETICS).

As is indicated in table 18, the effect of the alcohols on light-production is very similar to their effect on other life processes; they exert an inhibiting or anesthetic action which is perfectly reversible. If alcohol

TABLE 18.—*Effect of alcohols.*

Concentration of alcohol added to sea-water.	Light after—		
	10 min.	1 hour.	24 hours.
Methyl alcohol, 2m	−	−	−
HCH_2OH, 1.5 m	+	Very faint.	−
HCH_2OH, m	+	Faint.	−
HCH_2OH, m/2	+	+	+
HCH_2OH, m/3	+	+	+
Ethyl alcohol, m	−	−	−
CH_3CH_2OH, m/1.5	Very faint.	−	−
CH_3CH_2OH, m/2	+	Faint.	Faint.
CH_3CH_2OH, m/3	+	+	+
Propyl alcohol, m/3	−	−	−
$CH_3CH_2CH_2OH$, m/4	Very faint.	−	−
$CH_3CH_2CH_2OH$, m/6	Faint.	Very faint.	−
$CH_3CH_2CH_2OH$, m/8	+	Faint.	−
$CH_3CH_2CH_2OH$, m/15	+	+	+
Isobutyl alcohol, m/10	−	−	−
$(CH_3)_2CHCH_2OH$, m/12	Very faint.	−	−
$(CH_3)_2CHCH_2OH$, m/16	+	Very faint.	−
$(CH_3)_2CHCH_2OH$, m/20	+	+	−
$(CH_3)_2CHCH_2OH$, m/24	+	+	+
Amyl alcohol, m/20	−	−	−
$C_2H_5CH_3CHCH_2OH$, m/40	−	Very faint.[1]	+[1]
$C_2H_5CH_3CHCH_2OH$, m/80	Very faint.	Very faint.[1]	+[1]
$C_2H_5CH_3CHCH_2OH$, m/160	Faint.	+[1]	+
$C_2H_5CH_3CHCH_2OH$, m/320	+	+	+
Capryl alcohol, m/400	−	−	−
$CH_3(CH_2)_6CH_2OH$, m/800	Very faint.	Very faint.	−
$CH_3(CH_2)_6CH_2OH$, m/1600	Faint.	Faint.	−
$CH_3(CH_2)_6CH_2OH$, m/3200	Faint.	Faint.	+[1]
$CH_3(CH_2)_6CH_2OH$, m/6400	+	+	+
Sea-water	+	+	+

[1]Probably due to evaporation of alcohol.

solutions containing bacteria which have stopped emitting light are diluted with sea-water, light-production again begins. As with other tissues, the higher the alcohol in the series the greater anesthetic power it has.

The effect of a number of other substances was studied in a very rough way, namely, by adding a small quantity of the substance to a sea-water emulsion of the bacteria in test-tubes and then shaking

the tubes. With toluol, benzol, ether, chloroform, carbon disulphide, carbon tetrachloride, and ethyl butyrate the light was found to disappear almost immediately; with tannin, chloral hydrate, vanillin, and sodium glycocholate the light had disappeared in the course of an hour, while saponin, amygdalin, and sodium taurocholate had no effect. It is surprising that saponin has no effect on luminous bacteria when we consider its great cytolytic power on other forms in very small concentration.

EFFECT OF POTASSIUM CYANIDE.

As is the case with other luminous forms, KCN has a surprisingly small effect on the light-production of luminous bacteria as compared with its effect in supressing certain oxidations of many animals. The results are expressed in table 19.

TABLE 19.—*Effect of potassium cyanide.*

Concentration of KCN in sea-water.	Light after—		
	10 min.	1 hour.	24 hours.
m/40	−	−	−
m/80	Faint.	Very, very faint.	−
m/160	Faint.	Very faint.	−
m/320	Fair.	Fair.	−
m/640	+	+	−
m/1280	+	+	−
m/2560	+	+	+
Sea-water	+	+	+

In m/40 KCN the light disappears in 6 minutes, and if the solution is now diluted with sea-water the light returns. The effect is therefore an inhibition and reversible.

SUMMARY OF RESULTS FROM LUMINOUS BACTERIA.

1. Luminous bacteria which have been rapidly dried over calcium chloride in a vacuum will phosphoresce if moistened with oxygen-free water. Drying does not kill all bacteria, but does kill most of them. Hence phosphorescence does not depend on the living cell.

2. Dried bacteria, if finely ground with sand, will no longer phosphoresce when moistened. None of the ground bacteria can grow. Phosphorescence does depend upon the integrity of some structure in the cell.

3. Dried bacteria extracted with ether or toluol will still phosphoresce if moistened and may develop colonies on a suitable culture medium. Consequently neither ether nor toluol destroy the photogen.

Bacteria in oxygenated sea-water to which ether or toluol is added stop phosphorescing, presumably because the photogenic substance is rapidly oxidized and used up when the bacterial cell is cytolyzed.

Bacteria in oxygen-free sea-water do not glow, but will glow if oxygen is admitted, even after a period of 24 hours. Bacteria in oxygen-free sea-water to which toluol or ether is added will not glow if oxygen is readmitted after 15 minutes. Hence the phosphorescent substance undergoes decomposition in the absence of oxygen, a decomposition not due to the toluol (compare the first statement in section 3), but probably due to the action of some other substance.

4. Moist luminous bacteria to which oxygenated distilled water is added cease glowing, presumably because the photogen is rapidly oxidized and used up when the bacterial cell is cytolyzed.

Moist bacteria to which oxygen-free distilled water is added will not glow even momentarily if oxygen be readmitted after 15 minutes, a result again pointing to instability of the photogen when the cell-structure is affected by cytolysis.

5. Dried bacteria placed in oxygenated sea-water phosphoresce momentarily, but if dried bacteria stand in contact with oxygen-free sea-water for 15 minutes, no phosphorescence occurs when the oxygen is admitted. Again (as in sections 3 and 4) the photogen has decomposed. It is, therefore, impossible to extract a phosphorescent substance from bacteria with oxygen-free aqueous solvents.

6. Fat solvents extract nothing which will phosphoresce from the dried bacteria. Some of the bacteria survive and will grow after such extraction. Boiling alcohol, cold acetone, and ethyl butyrate destroy the power to phosphoresce.

7. Dried bacteria do not lose their power to phosphoresce after 24 hours' extraction with cold absolute alcohol, but moist bacteria (centrifuged) treated with 50 volumes of absolute alcohol, and then dried rapidly, will not again phosphoresce if moistened.

8. All attempts to separate the photogenic substance of luminous bacteria into photogenin and photophelein have failed, due possibly to the small amounts of these substances present at any one time.

9. A photophelein can be prepared by absolute alcohol which will give a faint light with firefly photogenin, but attempts to obtain light with firefly photophelein and bacterial photogenin prepared in various ways have failed.

10. The upper temperature-limit for luminescence is 38°; the lower is −11.5°. Bacteria heated to 38° and cooled give only a faint light, but bacteria will glow strongly if first cooled to liquid-air temperature and then raised to room temperature.

11. Oxidases for guaiac, *a*-napthol, para-phenylen diamine, phenol, phenolphthalin, pyrogallol, or indo-phenol formers, if present, are present in an endoenzyme condition. Catalase exists in luminous bacteria.

12. Luminous bacteria can not live if the osmotic pressure of the medium fall below a certain value or if the salt-content of the medium fall below a certain value.

13. Luminous bacteria will live for over 24 hours in pure m/2 NaCl or any sea-water salt mixtures containing both monovalent and divalent cations. They will live for over an hour in m/2 KCl, but the light disappears instantly in m/3 $CaCl_2$ or m/3 $MgCl_2$.

14. Luminous bacteria will just live for slightly over an hour in n/8,000 HCl, n/4,000 valerianic acid, and will live for over 24 hours in n/1000 NaOH and n/500 methyl amine. The best light is given on the alkaline side of neutrality, about the alkalinity of sea-water.

15. The homologous aliphatic monohydric alcohols produce true reversible inhibition or anesthesia of light-production. The concentrations which inhibit light-production in 10 minutes are: methyl alcohol, 2 m; ethyl, m; propyl, 0.25 m; isobutyl, 0.08 m; amyl, 0.025 m; capryl or octyl, 0.002 m.

16. KCN has very little effect on light-production. The light is only slightly affected in m/320 concentration in one hour.

STUDIES ON A SQUID, WATASENIA SCINTILLANS.

GENERAL CHARACTERISTICS OF LIGHT-PRODUCTION.

The structure of the luminous organs and the habits of this form have been well described by Ishikawa (31) and Sasaki (32). The organs are found on the tip of the ventral arms (3 in number), on the eyeball (5 in number), and over the ventral surface of the body (many). Only those of the arm-tips are brilliant enough to make chemical studies feasible. Their light is a brilliant bluish white like that of *Cypridina*. The intensity is high, but is no doubt due in large part to the reflectors present in the gland. The luminous material is burned within the cells. The animal is a deep-sea form, coming to the surface near shore to breed in certain bays of the Japanese coast, notably Toyama Bay. The surface-water of this bay is brackish and the animals will not live in it for even one hour. The deeper waters of the bay are more salty and in these the squid will live for a longer time if the water is changed to remove the poisonous ink. If oxygen be forced through fine pores into the water, the squid, as Professor Shoji, of Kyoto University, has shown, will live perhaps 8 hours, but this is the maximum. It will be seen, therefore, that the animal is extremely delicate and it is not surprising to find it of little value for chemical research. The arm-tip light-organs respond only to stimulation and the response disappears in 5 to 10 minutes after the animals are removed from sea-water. If the arms are cut off and placed in sea-water the light-producing substance entirely disappears in 30 to 60 minutes, as we can determine by grinding in a mortar. No light appears. The arm-tip light-organs become exhausted very rapidly if continually stimulated, and the light disappears very quickly after the organs have been ground in a mortar with water or sea-water.

The light of the skin-organs scattered over the ventral surface lasts much longer, and even after removal from the sea-water for 1 hour they will give a light which is more yellowish if stimulated electrically. The response is localized in the region about the electrodes.

The eye-organs are difficult to stimulate. Their light is weak and similar to that of the skin-organs.

The squid are caught at night in enormous numbers during April, May, and June by the fishermen, and Professor C. Ishikawa informs me that if brought into a dark room in the daytime they give light. The squid for this experiment were caught at 3 a. m. and tested about 8 a. m.—*i. e.*, 3 or 4 hours after daylight had appeared. There is no inhibiting influence of daylight on light-production, as described by Peters (4) for *Ctenophores* or Moore (5) for various luminous plankton forms. Neither have I observed any such influence of light with *Monocentris, Cypridina, Cavernularia, Pennatula,* or *Noctiluca.*

PHOTOGENIN AND PHOTOPHELEIN.

An extract presumably containing *Watasenia* photogenin can be prepared in the usual way by grinding the arm-tip light-organs in a mortar. As already mentioned, the light fades very rapidly, and it is impossible to again obtain light by mixture with the boiled-tissue extract of the squid (or with tissues heated to temperatures below boiling), which presumably should contain photophelein. Many attempts were made to obtain the photogenin-photophelein reaction, but all failed. This result is probably due to the fact that *Watasenia* contains more photophelein than photogenin, so that all the photogenin is used up when the arm-tip organs are ground. At least, *Watasenia* contains photophelein which will give light with firefly photogenin, whereas the firefly photophelein will give no light with *Watasenia* extracts which we might suppose to contain photogenin (p. 198).

STUDIES ON A FISH, MONOCENTRIS JAPONICA.

Monocentris japonica is a shallow-water marine fish having two light-organs—one on each side of the under surface of the tip of the lower jaw. The light is a weak glow with the faintest bluish tinge and is continuous and of almost unvarying intensity. In this respect it differs from most fishes, whose light is a series of flashes, the result of stimulation (Oshima, 33). The steady glow of the organs of *Monocentris* can be observed if the fish is removed from daylight to a dark room. The structure of the organ has never been investigated.

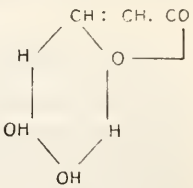
If the organ is ground in a mortar, the light lasts for some time, but does not reappear if mixed with *Monocentris* photophelein. Neither was light obtained by mixing *Monocentris* photogenin and *Cypridina* photophelein or *vice versa,* but as material was scarce, only one experiment was performed, and these results must not be considered conclusive.

STUDIES ON OXIDATIVE CHEMILUMINESCENT REACTIONS WITH SUBSTANCES OF DEFINITE COMPOSITION.

HISTORICAL.

Since the researches of Radziszewski in 1877 (34) and 1880 (35) on lophin and various oils, alcohols, and aldehydes, we have come to recognize an ever-increasing number of substances which will luminesce under appropriate conditions at temperatures approaching those compatible with life. Lophin (triphenylglyoxaline) gives an especially good light with alcoholic potash at 60° C., as do many essential oils. Trautz (36) has added other substances to the list of chemiluminescent reactions. R. Dubois (37) discovered the luminescence of esculin, a glucoside obtained from horse-chestnut bark and chemically a combination of glucose and esculetin.

These substances all require fairly strong alkali, although I have a sample of bergamot oil which gives a beautiful light when mixed with n/10 NaOH at 20° C. and a faint light at 5° C. At 20° C. a faint light appears with n/80 NaOH. On warming, the light is brighter. n/80 NaOH is, however, incompatible with life. Lophin and esculin will not luminesce except with alcoholic NaOH or KOH.



Esculetin.

McDermott (38) has described the production of light if to urine is added some 99 per cent KCN or potassium formate or formaldehyde and then a strong alkaline solution of H_2O_2. The substance in urine responsible for this effect is unknown. He describes light-production upon adding strong alkaline H_2O_2 to the cleavage products of Witte's peptone or glue made with alkali in absence of oxygen and to which a little commercial formaldehyde is added. (Note that the light is connected in these cases with substances found in organisms.)

Ville and Derrien (39) made an interesting step forward when they showed that lophin would luminesce with blood and H_2O_2, and Dubois (10) has recently discovered that esculin also will give light with blood and H_2O_2. The blood contains an oxygen carrier, hemoglobin, which accelerates the oxidation of the lophin and esculin. I can confirm the statement of Dubois and of Ville and Derrien, and find, also, contrary to Dubois, that esculin will give light with $KMnO_4$ and H_2O_2. It is best to keep the temperature about 60° C. I found also that esculin would give light with $FeCl_3+H_2O_2$, but not with $FeCl_3$, or $KMnO_4$ or H_2O_2 alone, nor with plant extracts rich in oxidases (turnip, potato, and horseradish root), either with or without H_2O_2, and that blood extract still retains its power to produce light with lophin after boiling. This is not surprising, as blood will also oxidize guaiac after boiling and upon addition of H_2O_2. Ozonized turpentine can take the place of H_2O_2 in oxidation of esculin.

Trautz (36) has shown that if we mix 35 c.c. of a 50 per cent K_2CO_3 solution, 35 c.c. of 10 per cent pyrogallol, and 35 c.c. of 35 per cent formaldehyde, and to this mixture add 50 c.c. of 30 per cent H_2O_2, a glow occurs, accompanied by much foaming. I can confirm this result, and find in addition that when the glow has died, if we add some $KMnO_4$, a reddish glow again appears. The tube becomes perceptibly warm.

It is not necessary to use such strong reagents to obtain light from oxidation of pyrogallol, however, for a very weak solution of pyrogallol will give a bright light if oxidized under the proper conditions. The oxidation of a mixture of pyrogallol + H_2O_2 by the vegetable oxidases occurs with the production of light. The reaction is highly interesting and remarkable for the following reasons: Perceptible light is produced with the concentration of pyrogallol m/32,000—*i. e.*, 1 part in 254,000 parts of solution; a faint light is produced at 0° C. and a bright light at 10° C.; KCN inhibits the reaction in m/2,000 concentration; boiling destroys the oxidase, and the power of producing light just as boiling destroys the light-producing power of organisms.

THE CHEMILUMINESCENCE OF PYROGALLOL.

It has been known for a long time that pyrogallol takes up oxygen from the air in presence of alkali and is converted into brown oxidation products of unknown composition. The depth of brown coloration (and consequently the extent of oxidation) depends on the concentration of alkali added. I have never under any conditions obtained light during this oxidation, although concentrations of alkali ranging from n to n/6,000 NaOH, both with and without H_2O_2, have been used. It has also been long known that pyrogallol will turn brown with H_2O_2 and some oxidizing enzyme from organisms. Purpurogallin, a substance of doubtful composition ($C_{11}H_8O_5$?), is said to be formed and the reaction has been used in studying oxidizing enzymes quantitatively. Only if H_2O_2 be present will this reaction produce light. It is similar to the oxidation of gum guaiac to guaiacum blue, but I find that guaiacum blue can be formed under many conditions when pyrogallol can not be oxidized with light-production. Thus hydrogen peroxide is necessary for light-production, even when potato-juice is used as the oxidase solution—*i. e.*, even when a juice is used containing an oxidase which will oxidize guaiac tincture directly (without addition of H_2O_2). It is then strictly a peroxidase which is responsible.[1] Blood also gives

[1]The difference between potato-juice, which oxidized guaiac without addition of H_2O_2, and turnip-juice, which requires the addition of H_2O_2, appears to lie in the fact that the potato-juice contains a substance which oxidizes spontaneously in presence of oxygen to a peroxide, while the turnip-juice does not. Consequently, we must add a peroxide to the turnip-juice. This spontaneously oxidizable substance was called oxygenase by Bach and Chodat. Both juices contain peroxidase. The "direct oxidases" (of potato) consist, then, of peroxide (oxygenase) + peroxidase, while the "indirect oxidases" (of turnip) are peroxidase alone. In this paper oxidase is used as a general name for an oxidizing enzyme and peroxidase for an enzyme transferring oxygen from a peroxide to an oxidizable substance. (See the account of this subject in Bayliss's Principles of General Physiol., 1915, 584; also see the monographs of Kastle (42), Warburg (Ergeb. d. Physiol., XIV, 253, 1914), Batelli and Stern (Ergeb. d. Physiol., XII, 96, 1912), and Bach and Chodat in Abderhalden's "Biochemische Arbeitsmethoden. III, No. 1, p. 42. Literature lists are given in the first three monographs.)

the reaction if H_2O_2 is present. Catalase takes no part, and may be destroyed by heating the potato-juice to 60°.

Thinking that this reaction might throw some light on luminescence in organisms, I have studied it in some detail, with especial reference to the substances which are known to affect light-production in organisms. It is the oxidases, similar to those which blue guaiac, that are concerned in the light-production of pyrogallol and potato-juice, but, as we shall see, many inorganic catalyses are also able to oxidize pyrogallol with light-production. It may be noted in passing that although light can be produced at 0° C., a considerable amount of heat is given off, and in this respect the oxidation differs markedly from those giving light in organisms.

TABLE 20.

Concentration of pyrogallol in a mixture which contains (after mixing) 50 per cent potato-juice and 0.93 per cent H_2O_2.	Character of light from the mixture.	Concentration of pyrogallol in a mixture which contains (after mixing) 0.93 per cent H_2O_2 and 0.5 per cent blood-extract.	Character of light from the mixture.
m/4	Faint.	m/40	Faint.
m/8	Fair.	m/80 to m/8000	Bright.
m/16 to m/8192	Bright.	m/16000	Fair.
m/16384	Fair, faint.	m/32000	Faint.
m/32768	Very faint.	m/64000	Very faint, none.
m/65536	None.	m/128000	None.

In studying the reaction I have used mostly pure potato-juice (*i. e.*, the pressed, unfiltered extract of grated potatoes, strained through cheese-cloth and decanted from the starch-grains) or a 1 per cent ox-blood extract (*i. e.*, 1 gram of dried defibrinated ox blood to 99 parts water) added to an equal volume of a half-and-half mixture of m/100 pyrogallol and 3 per cent H_2O_2 (Merck's perhydrol). The experiments will be given in the form of tables or data under the following heads:

1. Concentration of the three substances necessary for light-production.
2. Substances taking the place of blood or potato oxidase, pyrogallol, and H_2O_2.
3. Effect of temperature on the reaction.
4. Effect of KCN, NaOH, and HCl.
5. Effect of fat-solvent anesthetics.
6. Is the oxidase a catalyzer?

1. CONCENTRATION OF THE THREE SUBSTANCES NECESSARY FOR LIGHT-PRODUCTION.

In general, the more concentrated the blood or potato-juice the brighter the light, and the more concentrated the pyrogallol (to a limit) the brighter the light, and the longer it lasts. Too strong pyrogallol precipitates the blood-extract and potato-juice, the former in weaker concentration than the latter (see table 20). In about m/8000 concentration the pyrogallol gives a bright light with potato-juice, and

increasing the concentration above this does not increase perceptibly the brightness.

Each substance must be present in a definite small concentration to produce light. This is m/32,000 for pyrogallol, 0.025 per cent for H_2O_2, 0.1 per cent for dried blood, and 5 per cent for potato-juice—*i. e.*, 5 parts pure potato-juice to 95 parts water.

Note also, from tables 21 and 22, that very often the guaiac reaction can be obtained when, under similar conditions, no light-production by pyrogallol takes place.

TABLE 21.

Concentration of potato-juice in a mixture containing (after mixing) m/400 pyrogallol and 0.75 per cent H_2O_2.	Character of light from the mixture.	Blue color with tincture of guaiac.	Concentration of blood-extract in a mixture containing (after mixing) m/400 pyrogallol and 0.75 per cent H_2O_2.	Character of light from the mixture.	Blue color with tincture of guaiac
25 per cent.....	Bright.....	Blue.	0.5 per cent.....	Bright.....	Blue.
10 per cent.....	Faint.....	Blue.	0.25 per cent....	Faint.....	Blue.
5 per cent.....	Very faint..	Faint blue.	0.05 per cent....	Very faint..	Blue.
2.5 per cent....	None.....	Faint blue.	0.025 per cent...	None.....	Blue.
0.5 per cent....	None.....	Very faint blue.	0.01 per cent....	None.....	Blue.

2. SUBSTANCES TAKING THE PLACE OF OXIDASE, PYROGALLOL, AND H_2O_2.

Oxidase.—Any plant extract containing strong oxidases, such as occur in turnips, sweet potatoes, horseradish, etc., or any blood containing hemoglobin, will give light with pyrogallol and H_2O_2. Apple-juice, which oxidizes tannin, gives no light. Extracts of many invertebrates were tried (including insects, molluscs, annelids, crustaceans, and echinoderms), but only those of *Chiton*, a tubicolous annelid (*Laonome japonica*), and some polychætes have thus far been found to give light. The extract must be concentrated and the light-production is probably due to hemoglobin, as it is obtained in many cases after the extract is boiled. The substances in *Chiton* seem to come from red filaments near the mouth (muscles ?). Contrary to my earlier experiments, which were performed with too-dilute extracts, I find that the blood of a squid (*Sepia esculenta*) (containing hemocyanin) and the orange blood of the Japanese lobster (*Panulirus japonica*), will also give light, and the light is somewhat brighter if the blood has previously been boiled. The extracts of luminous animals never gave light with pyrogallol+H_2O_2. Among these, bacteria, an annelid (*Chætopterus*), a squid (*Watasenia scintillans*), a crustacean (*Cypridina*), a protozoan (*Noctiluca*), a pennatulid (*Cavernularia*), and the firefly have been tried.

Inorganic substances which give light may be divided into three classes: (1) insoluble precipitates, (2) colloidal metals, (3) solutions of various salts. H_2O_2 must always be added. The first two classes probably act as true inorganic catalyzers.

Among the substances of class (1) may be mentioned MnO_2, mixtures of $Mn(OH)_2$ and $Mn(OH)_3$, H_2MnO_3, $Fe_2Fe(CN)_6$, and Ag_2O. All give light. I have observed no light with boneblack, PbO_2, Ag_2CrO_4, $PbCrO_4$, $BaCrO_4$, $Ag_4Fe(CN)_6$, $Pb_2Fe(CN)_6$, $Zn_2Fe(CN)_6$, $Cu_2Fe(CN)_6$, HgO, $Cu(OH)_2$, Cu_2O, CuO, FeO, iron filings, Hg_2Cl_2, Cr_2O_3, $Cr(OH)_3$, $Fe(OH)_3$, Pt black, and silica.

Among the group (2), a silver sol protected by protein so as to be fairly concentrated gave a good light. Concentrated Pt sol and Au sol also gave light. Colloidal $Fe(OH)_3$ and copper oxide gave negative results.

TABLE 22.

Concentration of Merck's special H_2O_2 in a mixture containing (after mixing) m/400 pyrogallol and 50 per cent potato-juice.	Character of light from the mixture.	Blue color with tincture of guaiac.
0.47 per cent.....	Bright.....	Blue.
0.23 per cent.....	Fair.....	Blue.
0.11 per cent.....	Weak.....	Blue.
0.05 per cent.....	Faint.....	Blue.
0.025 per cent.....	Very faint..	Blue.
0.012 per cent.....	None.....	Blue.

The following salts gave the best light in the concentrations indicated: m/10 to m/20 $K_4Fe(CN)_6$, m/100 $K_2Cr_2O_7$, m/50 K_2CrO_4 (the light appears only after a long time), m/100 $KMnO_4$, m/100 CrO_3, m/10 $FeCl_3$. The following gave no light: $K_2S_2O_8$ (m/10 to m/320), $KClO_3$ (m/10), $K_3Fe(CN)_6$ (m/10 to m/1000) $MnCl_2$ (m/10), $AgNO_3$ (2 per cent), $CuSO_4$ (m/10), and bromine water. The mechanism of the action of these diverse substances will be considered in a subsequent paper.

Peroxide.—Only H_2O_2 will give light with pyrogallol and an oxidase. Na_2O_2, benzoyl hydrogen peroxide, quinone, BaO_2, or ozonized turpentine do not, although they give a strong guaiac reaction with the peroxidase of turnip-juice. We must also add H_2O_2 to potato-juice, which already contains an organic peroxide whose oxygen may be transferred to guaiac. It is no doubt in too small a concentration to give light with pyrogallol.

Oxidizable substance.—Many of the amino- and hydroxy-phenols are rapidly oxidized by potato-juice + H_2O_2, but pyrogallol was the only one which gave light. The following were tried and all gave colored oxidation products: phenol, tricresol, pyrocatechin, resorcin, hydrochinon, orcinol, *a*-napthol, ortol, anilin, *o* and *p* toluidin, *a*-napthylamin, guaiacol, para-phenylen diamine, amidol, and pyramidon. Phloroglucin, *B*-napthol, and *B*-napthylamin were not oxidized.

Among the glucosides, vanillin, amygdalin, phloridizin, salicin, saponin, and esculin gave no light with potato-juice, either at 20° or 60° C.

3. Effect of Temperature on the Reaction.

A very faint light is produced when pyrogallol + H_2O_2 at 0° C. is mixed with potato-juice at 0° C. The light is much brighter at 5° and quite bright at 10° C. Pyrogallol + H_2O_2 mixed with blood (1 per cent) gives no light at 0° C., but a very faint light at 2° C.

High temperatures destroy the pyrogallol oxidase of potato-juice. This occurs between 80° and 85° C., as indicated in table 23. The potato-juice was rapidly heated to the temperatures 50°, 55°, 60° C., etc., then cooled rapidly in running water. The destruction of catalase, the formation of a precipitate in the potato-juice, and the effect on its power to blue guaiac alone, guaiac + H_2O_2, and guaiac + ionized turpentine, are also recorded. Similar results were obtained with turnip-juice. Blood does not lose its power to oxidize pyrogallol + H_2O_2 with light-production or guaiac + H_2O_2, even after 10 minutes' boiling.

Table 23.

Potato-juice heated to—	Light-production with pyrogallol + H_2O_2.	Bluing of guaiac.	Bluing of guaiac + H_2O_2.	Bluing of guaiac + ozonized turpentine	Effect on catalase; liberation of O from H_2O_2.	Formation of precipitate in potato-juice.
50 degrees.....	Bright.....	Blue.....	Blue...	Blue....	+++.	None.
55 degrees.....	Bright.....	Blue.....	Blue...	Blue....	+.....	None.
60 degrees.....	Bright.....	Blue.....	Blue...	Blue....	None..	None.
70 degrees.....	Faint.....	Slight bluing.	Fair bluing.	Blue....	None..	Precipitate.
80 degrees.....	Very, very faint.	Very slight bluing.	Slight bluing.	Blue....	None..	Precipitate.
85 degrees.....	None.....	None.....	None..	Blue....	None..	Precipitate.
90 degrees.....	None.....	None.....	None..	Blue....	None..	Precipitate.
100 degrees.....	None.....	None.....	None..	Slight...	None..	Precipitate.
100 deg. (10 min.)	None.....	None.....	None..	None...	None..	Precipitate.

Note from table 23 that the pyrogallol and guaiac oxidases in presence of H_2O_2 are both destroyed between 80° and 85° C., but that with ozonized turpentine the juice heated to 100° C. still blues guaiac faintly. The natural peroxide (oxygenase) of potato-juice, which disappears on standing, is destroyed more readily than the peroxidase. Catalase is destroyed between 55° and 60° C. before a precipitate has appeared, whereas peroxidase is still active after a precipitate has appeared.

4. Effect of KCN, NaOH, and HCl.

In testing the effect of these substances, equal parts of (1 c.c. m/160 pyrogallol + 1 c.c. 3 per cent H_2O_2) and (potato-juice + KCN or NaOH or HCl) were mixed. Table 24 gives also the effect of catalase on the bluing of guaiac + H_2O_2, and the color of indicators added to determine the amount of acid or alkali uncombined with protein. Neutral red is red in neutral or acid solution, yellow in alkalies. "Di-methyl" is yellow in neutral or alkaline solution, red in free acid solution.

The concentration of acid and alkali added give, of course, no idea of the H-ion concentration in the potato-juice. The oxidase acts best in the region of neutrality or slight alkalinity, and Reed (40) finds by hydrogen electrode measurements that the oxidase of potato is inhibited in a $C_H 1.5 \times 10^{-4}$. The effect of the acid is reversible unless it has acted too long.

Note from table 24 that KCN affects the pyrogallol peroxidase in very weak concentration (m/2,000), but is not so effective in destroying catalase or preventing the bluing of guaiac. NaOH and HCl are not so toxic. As we might expect, the HCl is the more toxic of the two.

TABLE 24.

Substance.	Concentration in potato-juice.	Light-production with pyrogallol + H_2O_2.	Liberation of O from H_2O_2.	Bluing of guaiac + H_2O_2.	Color of indicator.	Indicator.
KCN...	m/20	None	+	None	Yellow	Neutral red.
	m/40	None	+	Very faint blue.	Orange	
	m/80	None	++	Fair blue	Orange	
	m/160	None	++	Fair blue	Red	
	m/320	None	++	Fair blue	Red	
	m/640	None	++	Fair blue	Red	
	m/1280	None	+++	Blue	Red	
	m/2560	Very faint	+++	Blue	Red	
	m/5120	Faint	+++	Blue	Red	
	m/10240	Fair	+++	Blue	Red	
	m/20480	Bright	+++	Blue	Red	
NaOH...	m/20	None	+	None	Yellow.	
	m/40	None	+++	Faint blue	Yellow.	
	m/80	Faint	+++	Blue	Yellow orange.	
	m/160	Bright	+++	Blue	Red orange.	
	m/320	Bright	+++	Blue	Red.	
HCl	m/20	None	None	None	Red orange	Dimethyl-amino azobenzol.
	m/40	None	None	Faint blue	Yellow	
	m/80	None	None	Faint blue	Yellow	
	m/160	Fair	+	Blue	Yellow	
	m/320	Bright	+++	Blue	Yellow	
	None	Bright	+++	Blue	Yellow	

5. EFFECT OF FAT-SOLVENT ANESTHETICS.

Saturation of the potato-juice or blood with ether or chloroform does not affect its power to produce light with pyrogallol + H_2O_2. A sample of horseradish-root extract preserved 45 days under ether gave a good light when mixed with pyrogallol + H_2O_2.

6. IS THE OXIDASE A CATALYZER?

That oxidases in general are not catalyzers, but are used up in the reaction has been proven by the work of Kastle (16), who found that the amount of phenolphthalein oxidized is proportional to the quantity of blood present, and of Bach and Chodat (41 and 42), who found that the amount of pyrogallol oxidized to purpurogallin is proportional to the amount of peroxidase and also to the amount of H_2O_2 present,

but independent of the quantity of pyrogallol, providing the pyrogallol is present in excess of the quantity capable of being oxidized by peroxidase and H_2O_2. Melanin formation by tyrosinase is also proportional to the amount of tyrosinase present (**42**).

The peroxidase, therefore, transfers the O of H_2O_2 to the pyrogallol and the H_2O_2 pyrogallol, and peroxidase are all changed—*i. e.*, used up in the reaction. If we represent the peroxidase by P and the pyrogallol by B the reaction must take place as follows:

$$\begin{aligned} P \quad &+2H_2O_2 = PO_2 + 2H_2O \\ PO_2 &+ B \quad = PO + BO \end{aligned} \qquad (1)$$

and not as

$$\begin{aligned} P \quad &+2H_2O \; = PO_2 + 2H_2O \\ PO_2 &+2B \quad = P \quad +2BO \end{aligned} \qquad (2)$$

Note that in the first equations the peroxidase (P) is used up and in the second equations it acts as a true catalyzer and is regenerated again.

That the peroxidase of turnip-juice is used up so far as its power to cause light-production in pyrogallol is concerned is indicated in the following experiment:

If 1 c.c m/10 pyrogallol+4 c.c. H_2O_2 (3 per cent) is mixed with 5 c.c. turnip-juice heated to 80° (to destroy the catalase and weaken the peroxidase), a very faint light is produced. A small amount of peroxidase is, therefore, present. The mixture is allowed to stand for 24 hours at 22° C. and then fresh turnip-juice is added. A good light results, showing that the pyrogallol has not been changed by a small amount of peroxidase when a long time has been allowed for the reaction to proceed. The pyrogallol was turned a light brown by the small amount of peroxidase, and this color had not deepened in 24 hours, but did deepen immediately when the additional quantity of peroxidase was added. A small quantity of "enzyme" can not, therefore, transform an indefinite amount of pyrogallol. A similar result was obtained with a weak hemoglobin solution, 0.1 per cent dried blood-extract.

That a large quantity of peroxidase can be used up by addition of successive amounts of pyrogallol—*i. e.*, by "titrating" the peroxidase—can be shown as follows:

To 10 c.c. potato-juice heated to 60° C. (to destroy catalase and consequent foaming due to liberation of O from H_2O_2) is added 2 c.c. of a mixture of equal parts m/10 pyrogallol and 3 per cent H_2O_2. Light results. When this has disappeared (about 2 to 3 minutes) 2 more cubic centimeters of the pyrogallol-H_2O_2 mixture are added and a very faint light results. On adding a further 2 c.c. no light appears. There is, however, in the final mixture plenty of pyrogallol+H_2O_2 to produce light, as may be shown by adding fresh potato-juice when abundant light appears. The peroxidase has been completely used up. A similar experiment with blood-extract gave a similar result.

SIGNIFICANCE FOR BIOLUMINESCENCE.

The reader has doubtless already noticed the similarity of the pyrogallol+H_2O_2-peroxidase reaction and the luciferin-luciferase reaction of Dubois. Just as Dubois supposed luciferin to be oxidized by luciferase, so we actually find pyrogallol+H_2O_2 oxidized by peroxidase. We now know that luciferin (photophelein) is not oxidized by luciferase (photogenin) but that Dubois's luciferase is actually the *source* of the light of luminous animals; so that the luminescence of pyrogallol turns out to bear only a superficial resemblance to that of organisms. Pyrogallol+H_2O_2 will give no light with either photogenin or photophelein, nor will peroxidase give light with either photogenin or photophelein, even if we add H_2O_2. However, it is interesting and important to know that certain substances found in plants and animals can assist in oxidizing other substances found in plants and animals with the production of light. The light produced in these cases is faint compared with the light produced by luminous organisms or compared with the light produced by substances from luminous organisms in a test-tube, but nevertheless it is light with very little heat, and perhaps a further survey will reveal the existence of definite compounds producing light in a manner more closely resembling that of *Cypridina*. As has been shown by Trautz (36) the spectrum of luminescent pyrogallol is continuous but shorter than the normal spectrum, so that there is marked similarity in the physical characteristics of the light of chemiluminescent substances and of luminous organisms.

SUMMARY OF STUDY OF CHEMILUMINESCENT REACTIONS.

1. Pyrogallol gives off a yellowish-white light about equal in intensity to a suspension of luminous bacteria when oxidized by blood or plant-juices (oxidases) in presence of H_2O_2.

2. The light is visible in very weak concentrations of pyrogallol, viz, m/32,000 or 1 part to 254,000 parts solution. H_2O_2 must be present in at least 0.025 per cent, blood in 0.1 per cent (dried defibrinated blood), potato-juice in 5 per cent (fresh pure juice), to give a just visible light.

3. Of many easily oxidizable hydroxy- and amino-phenols only pyrogallol gives light. $K_4Fe(CN)_6$, $KMnO_4$ or $FeCl_3$, $K_2Cr_2O_7$, K_2CrO_4, MnO_2, $Mn(OH)_2$+$Mn(OH)_3$, H_2MnO_3, $Fe_2Fe(CN)_6$, Ag_2O, and colloidal Pt, Au, and Ag will all give light with pyrogallol. Extracts of invertebrates, except those of *Chiton*, a few marine annelids, and the blood of a squid (*Sepia*) and a marine crayfish (*Panulirus*), do not give light. Na_2O_2, BaO_2, PbO_2, benzoyl H_2O_2, quinone, or ozonized turpentine will not take the place of H_2O_2.

4. Light is produced at 0° C. and a bright light at 10° C. The oxidase of plant-juices is destroyed between 80° and 85° C.

5. KCN inhibits the light-production in very weak concentration, m/1280 to m/2560. NaOH inhibits in n/40 and HCl in n/80 concentration, but these figures give no idea of the H-ion concentration.

6. Ether and chloroform have no effect on the light-production.

7. The oxidase is not a true catalyzer, but is used up in transferring oxygen from H_2O_2 to the pyrogallol.

8. The reaction has nothing to do with the production of light by luminous animals.

GENERAL CONCLUSIONS.

We may be very certain that in all luminous animals a definite substance undergoes chemical change, and if free oxygen is present light is produced. This oxidizable substance may conveniently be called photogenin. In some forms it is oxidized within the cell, in others without. In some forms the oxidation goes on continuously, in others only after stimulation, using the word "stimulation" in the same sense in which it is used in referring to muscle contraction. In some forms photogenin occurs as granules, in others it may be obtained in a solution which will pass the finest porcelain filters. In some forms we can demonstrate the existence of a second substance necessary for light-production, photophelein; in others this can not be demonstrated. There are definite quantitative relations between photogenin and photophelein, and in those animals in which the two substances can not be demonstrated they are possibly present in equivalent amount. Photogenin is colloidal and probably a protein; photophelein is crystalloidal and of wholly unknown composition. Neither of the two are soluble in fat solvents. Photogenin is found only in luminous glands, photophelein throughout the organism and in non-luminous animals. Photopheleins from different species of luminous or non-luminous animals will give light with various photogenins if the animals are nearly related, but only a very faint light if distantly related. The connection between photogenin and photophelein resembles most that between the zymase (=photogenin) and cozymase (=photophelein) of yeast-juice. Both of the former substances must be present for the fermentation of sugar by yeast, just as both of the latter must be present for the production of light by luminous animals. As regards destruction by heat and dialyzability even the properties of the two corresponding substances are similar. Here the parallel ceases, at least so far as our knowledge is concerned. It is possible that a mixture of photogenin and photophelein oxidizes a third substance, just as zymase-cozymase oxidizes sugar, but we know of no third oxidizable substance. We do know that whenever photogenin and photophelein in solution are exposed to free oxygen, light is produced and both substances disappear. This is the present extent of our knowledge and only additional experimental work can give us a more definite idea of the nature of these substances.

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IX.

A PHYSIOLOGICAL STUDY OF SPECIFIC GRAVITY AND OF LUMINESCENCE IN NOCTILUCA, WITH SPECIAL REFERENCE TO ANESTHESIA.

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By Ethel Browne Harvey.

INTRODUCTION.

The present study has been chiefly concerned with the specific gravity and the effects of anesthetics on the luminescence of *Noctiluca*. The effects of other substances—acids, alkalies, KCN, etc.—have also been studied, as well as the action of electricity, change of osmotic pressure, temperature, etc., in order to gain an accurate knowledge of the more general physiological aspects of luminescence in this form. Some observations on these subjects were made by Quatrefages in 1850, Krukenberg in 1887, and Massart in 1893, but most of the work is too old and the methods too crude to be of much value, since definite concentrations were not considered nor the results interpreted from the physico-chemical aspect.

The results were obtained at the Marine Biological Laboratory of the Imperial University of Tokyo, situated at Misaki, Japan, where the great abundance of *Noctiluca* offered unusual advantages. The animals occur in such quantities that a layer an inch thick on the surface of a large aquarium jar can soon be collected, and on dark nights the sea is extremely brilliant in any area where it is disturbed, especially where the waves lap the shore. It gives me pleasure to thank Professor I. Ijima for the facilities of the laboratory, and also Professor C. Ishikawa for his many kindnesses during my stay there.

LIGHT RESPONSE IN NOCTILUCA.

As is well known, noctilucas, when undisturbed and under perfectly normal conditions, are not luminous; but when disturbed or stimulated in any way, they give a bright flash of a distinctly bluish tinge, lasting only an instant. This is the normal light-response of *Noctiluca*. If, however, the animals are not in good condition, a faint, continuous glow issues from them; such a glow is usually preliminary to the death of the animals, and is also called forth by many experimental conditions. These two are the only light responses of the animals. There is never, under normal conditions, a rhythmic flashing, such as is characteristic of certain species of firefly, nor is it possible, by any chemical or physical means, to call forth a rhythmic flashing comparable with the rhythmic twitching of a muscle in pure NaCl.

CHANGE OF OSMOTIC PRESSURE.

Noctiluca can stand a considerable decrease in osmotic pressure without having its luminous response affected, as indicated in table 1. We may dilute the sea-water with fresh-water in any amount down to a proportion of half and half, and the animals still give a normal response—*i. e.*, they flash on stimulation. The response is not quite so bright as in the control in a half-and-half concentration nor in a mixture of 4 sea-water to 6 fresh-water, but the animals continue giving a normal response for 7 days. In the latter concentration the animals also give a steady glow for the first few minutes, owing doubtless to the dying condition of certain of them. If the amount of fresh water is still further increased, the animals do not flash on stimulation, but give a steady glow, lasting for 8 minutes in a mixture of 3 sea-water to 7

TABLE 1.—*Effect of diluting sea-water with fresh-water.*

Ratio of sea-water to fresh-water.	Luminous response.	Specific gravity.
9 to 1.....	Normal, 10 days (experiment discontinued).	Float.
8 to 2.....	Normal, 10 days (experiment discontinued).	Float.
7 to 3.....	Normal, 9 days.....	Float.
6 to 4.....	Normal, 9 days.....	Distributed through water 10 minutes; then float.
5 to 5.....	Normal, 7 days (poor).....	Most sink in 15 minutes; float after 20 minutes.
4 to 6.....	Normal, 7 days (poor), constant glow 3 minutes at start.[1]	Sink in 15 minutes; float after 1 hour.
3 to 7.....	Constant glow 8 minutes.....	Sink.
2 to 8.....	Constant glow 8 minutes.....	Sink.
1 to 9.....	Constant glow 5 minutes.....	Sink.
Fresh-water.	Constant glow 3 minutes.....	Sink.

[1]The constant glow at the start is without doubt due to the dying condition of some of the animals.

fresh-water and of 2 sea-water to 8 fresh-water; and for 5 minutes in 1 sea-water to 9 fresh-water, and for about 3 minutes in pure fresh-water. When the osmotic pressure is increased by concentrating the sea-water to half its volume, a steady glow is given for more than 20 minutes.

Especially interesting results have been obtained in this series of experiments with regard to specific gravity. Normally, noctilucas are less dense than sea-water, so that when placed in an aquarium jar they soon rise to the top, where they form a layer somewhat pinkish in color. Unlike the floating siphonophores, there are no air-bubbles, and unlike certain pelagic eggs, there are no large oil-drops in the animals, so that their lower specific gravity must be due to the fact that their salt-content is less than that of sea-water. When placed in sea-water concentrated to half its volume they shrink, and when the sea-water is diluted with fresh-water they swell. The plasma membrane, therefore, shows the usual semipermeability to the balanced

salts of sea-water—*i. e.*, impermeability to the salts and permeability to water. Out in the open bay, although most of the animals are at the surface on a calm night, many can be seen through a glass-bottom bucket well below the surface. Moreover, on windy days, they are not found at the surface, indicating that they have the power of increasing their specific gravity and sinking, owing to a temporary increase in permeability which allows the salts of sea-water to pass in or water to pass out. When noctilucas die they immediately sink, the permeability having been increased, causing an increase in specific gravity.

That these animals can also decrease their specific gravity is shown by the following facts. In all dilutions of sea-water with fresh-water down to 6 sea-water to 4 fresh-water, they rise immediately to the top, becoming and remaining somewhat swollen for more than 10 days. These dilutions are still of greater density than the animals. In a mixture of 6 sea-water to 4 fresh-water they remain distributed through the water for about 15 minutes, and then rise to the top; this mixture is of approximately the same density as the noctilucas. In a mixture of half sea-water and half fresh-water and also in a mixture of 4 sea-water to 6 fresh-water, the animals sink at first, their salt-content being now greater than that of the surrounding medium, but during the next hour they gradually swell and rise to the top. The process is wholly independent of movement of the tentacle. If water were merely absorbed by the animals until their concentration was the same as that of the surrounding medium, the animals would remain suspended through the liquid; but since they eventually float, they must keep on absorbing pure water until their salt-content and hence their specific gravity is again *less* than that of the new medium (5 sea-water to 5 fresh-water or 4 sea-water to 6 fresh-water), thus reestablishing their normal relation to their surrounding medium. This must involve the absorption of water against the osmotic pressure of the salts of sea-water, a condition contrary to physical laws. This regulatory mechanism is characteristic of living cells only and is not destroyed under any experimental conditions. Anesthetics, acids and alkalies, KCN, and pure salts of sea-water do not affect the regulation, except when they cause irreversible changes and death of the cells. It is quite probable that this peculiar type of osmo-regulation is not characteristic of noctilucas alone, but may be possessed by other marine plankton forms without gas-chambers which float and sink as occasion demands.

The water absorbed by noctilucas in mixtures of 5 sea-water to 5 fresh-water and 4 sea-water to 6 fresh-water accumulates in large vacuoles formed by strands of protoplasm pulling away from the cell-wall and forming membranes around the accumulated liquid. I have observed these vacuoles bursting or being expelled bodily from the cell. In these same concentrations of sea-water an interesting protoplasmic

fragmentation also takes place, secondary smaller masses of protoplasm being formed, sometimes isolated and sometimes connected with the main mass by strands of protoplasm. The vacuoles formed by *Noctiluca* in these mixtures of fresh-water with salt-water are perhaps comparable with the contractile vacuoles of *Paramœcium*, *Amœba*, and other fresh-water forms, where its function is likewise osmoregulatory. It is true that in general in salt-water protozoa no contractile vacuoles are present, since the osmotic pressure of sea-water balances that of the animal and no osmoregulatory mechanism is needed. When *Noctiluca* is put under conditions where it must rid itself of a certain amount of fresh-water, similar though less perfect vacuoles can be formed.

In still more dilute mixtures of sea-water and fresh-water, the animals absorb water until they burst and then shrink; they are killed and sink to the bottom. That this is an osmotic effect and not due to the mere dilution of salts is shown by diluting the sea-water with iso-

TABLE 2.—*Effect of m cane-sugar+sea-water.*

m cane-sugar to sea-water.	Luminous response.
m sugar.....	Normal response 2 hours; constant glow during last hour.
9 to 1.....	Normal response 2 days.
8 to 2.....	Normal response 4 days.
7 to 3–1 to 9....	Normal response 9 days (experiment discontinued).

tonic cane-sugar, thus diminishing the salt-content but not the osmotic pressure. After testing various concentrations of cane-sugar to determine which was isotonic, it was found that the animals responded normally longest in m cane-sugar, so that this was used to dilute the sea-water. In m cane-sugar in distilled water the animals give a normal response for 2 hours; this is accompanied during the last hour by a steady glow; but when sea-water is added to the m sugar in various proportions, the effect on the luminescence is only slight (table 2). When 3 parts sea-water are added to 7 parts m sugar or any greater proportion of sea-water is used, the animals give a normal response for more than 9 days. In mixtures of 2 sea-water to 8 sugar a normal response is given for 4 days, and in 1 sea-water to 9 sugar a normal response is given for 2 days. The noctilucas, therefore, like muscle and other cells, must be bathed by a certain minimal salt-content as well as surrounded by a fluid with a certain osmotic pressure. They behave in this respect like the luminous bacteria investigated by E. N. Harvey (1914).

SALTS OF SEA-WATER.

In any one of the pure salts of sea-water, made up in m/2 solution in distilled water, *Noctiluca* ceases to give any luminous response after 2 hours (table 3). $CaCl_2$ is the most toxic, no normal response being given, but a constant glow for 2 minutes. KCl is least toxic, a normal response being given for 2 hours. In NaCl and in $MgCl_2$ there is a normal response for an hour, and this is accompanied on first immersion in the salt by a constant glow which soon passes off, doubtless owing to some of the animals dying. In a mixture of NaCl with one of the other salts, in the proportion occurring in sea-water, the normal response lasts much longer than in any of the pure salts; a little under a day in NaCl+KCl, and 5 days in NaCl+$CaCl_2$ or NaCl+$MgCl_2$.

TABLE 3.—*Effect of salts of sea-water.*

Solution (in distilled water).	Luminous response.
m/2 NaCl	Normal, 1 hour (poor); momentary constant glow at start.[1]
m/2 KCl	Normal 2 hours.
m/2 $CaCl_2$	Constant glow 2 minutes.
m/2 $MgCl_2$	Normal 1 hour; constant glow 10 minutes at start.[1]
m/2 (100 NaCl+2.2 KCl)	Normal, $<$1 day.
m/2 (100 NaCl+2 $CaCl_2$)	Normal, 5 days.
m/2 (100 NaCl+10 $MgCl_2$)	Normal, 5 days.
m/2 (100 NaCl+2.2 KCl+2 $CaCl_2$)	Normal, 5 days.
m/2 (100 NaCl+2.2 KCl+10 $MgCl_2$)	Normal, 7 days.
m/2 (100 NaCl+2.2 KCl+2 $CaCl_2$+10 $MgCl_2$)	Normal, 10 days.
Same mixture+m/2300 NaOH	Normal, 10 days.
Same mixture+m/2300 HCl	Constant glow 15 minutes.

[1]The constant glow at the start is doubtless due to the dying condition of some of the animals.

In a mixture of NaCl+KCl+$CaCl_2$ a normal response is given for 5 days, and in NaCl+KCl+$MgCl_2$ for 7 days. With the four salts in a neutral or alkaline medium (+m/2300 NaOH), the response lasted for more than 10 days, when the experiment was discontinued. The addition of a small amount of acid (m/2300 HCl) to the four salts prohibits any normal response, causing a constant glow for 15 to 30 minutes. (See also under Acids.)

These results showing the injurious effects of pure salts on the luminous response of *Noctiluca* and the improvement on balancing with other salts are in agreement with the general work on the effect of balanced solutions on other physiological activities. The luminous bacteria, on the other hand, are independent of a balanced medium, although they must, like *Noctiluca*, have a certain small proportion of salt in the medium (E. N. Harvey, 1914).

ACIDS AND ALKALIES.

For ascertaining the effects of acids on the luminescence of *Noctiluca*, hydrochloric (a lipoid insoluble) and benzoic (a lipoid soluble) acids were used, the solutions being made up in a neutral artificial sea-water, m/2(100 NaCl+2.2 KCl+2 $CaCl_2$+10 $MgCl_2$), so as to avoid the disturbing presence of the buffer phosphates and carbonates.

Certain concentrations of these acids, n/2000 to n/4000 HCl and n/4000 C_6H_5COOH, cause a bright continuous glow lasting from 20 to 60 minutes (table 4). The glow in these solutions becomes gradually

TABLE 4.—*Effect of acids and alkalies.*

Solution in artificial sea-water.	Luminous response.	Color change in neutral red.
n/500 to n/1000 HCl	Momentary constant glow.	
n/2000 HCl	Constant glow 30 min. (bright, becomes brighter, then fainter).	
n/4000 HCl	Constant glow and normal response 1 hr.	
n/8000 HCl	Normal 6 days (poor); momentary constant glow at start.[1]	
n/16000 HCl	>6 days (exp. discontinued).	
n/500 to n/2000 benzoic	Momentary constant glow.	
n/4000 benzoic	Constant glow 20 min. (becomes brighter, then fainter).	
n/8000 benzoic	>6 days (poor) (exp. discontinued); momentary constant glow at start.[1]	
n/16000 benzoic	>6 days (poor); (exp. discontinued).	
n/125 NaOH (in Mg-free sea-water)	Constant glow 2 min	4 min.
n/250 NaOH (in Mg-free sea-water)	Constant glow 2 min	10 min.
n/500 NaOH	Normal response 30 min., then constant glow 30 min.[2]	1 to 1½ hr.
n/1000 to n/2000 NaOH	Normal >7 days (exp. discontinued)	None.
n/250 NH_4OH	Constant glow 30 seconds	25 sec.
n/500 NH_4OH	Constant glow 75 seconds	30 sec.
n/1000 NH_4OH	Constant glow 4 min. (faint)	1 to 2 min.
n/2000 NH_4OH	Normal 3 min., then constant glow 6 min.[2]	None.
n/4000 NH_4OH	Normal 5 days	None.
n/8000 NH_4OH	Normal >7 days (exp. discontinued)	None.

[1]The constant glow at the start is without doubt due to the dying condition of some of the animals.

[2]The normal response at the start is doubtless due to the delay in taking effect.

brighter, stays at a maximum for some time, and then becomes gradually fainter. In n/4000 HCl the animals also give a normal response—that is, they flash on stimulation while they are giving a steady glow. Whether it is certain animals in the solution that give the one response at the same time that others are giving the other, or whether all of the animals give both responses, it was not possible to determine. Stronger concentrations of these acids kill the animals almost instantly, while weaker concentrations have practically no effect.

The alkalies NaOH (lipoid insoluble) and NH_4OH (lipoid soluble) have an effect upon luminescence similar to that of the acids, except

that the constant glow is more faint and of shorter duration; n/125 to n/250 NaOH and n/250 to n/1000 NH_4OH (the first two solutions were made up in Mg-free sea-water, all the others in artificial sea-water) cause a constant glow lasting from 30 seconds to 4 minutes, and no normal response is given (table 4). In slightly weaker solutions, n/500 NaOH and n/2000 NH_4OH, a normal response is given for a short period, owing doubtless to a delay in penetration, followed by a constant glow.

In one series of experiments comparison was made of the penetration time of the alkali and its effect on luminescence. The comparison was made on the same lot of animals stained with neutral red, which is not harmful to them. One set was observed in the dark for the effect on luminescence, while the other set was watched simultaneously by an assistant for color-change. In the case of NaOH the color-change from red to yellow takes place only *after* all light-response has stopped, whether this be a steady glow or a normal response; NaOH, therefore, does not penetrate the cell until after luminescence has ceased. On the other hand, with NH_4OH the color-change takes place *before* the effect on luminescence has stopped—that is, the alkali penetrates the cell while it is still giving a luminous response. For example, in n/500 NH_4OH the color-change takes place in 30 seconds, while the constant glow lasts 75 seconds. These results tally with those of E. N. Harvey (1914), who found that in certain plant and animal cells NaOH penetrates the cell only after its death, while NH_4OH penetrates before the death of the cell. The effect of alkalies on light-production is therefore brought in line with its effect on muscle-contraction, protoplasmic rotation, and other physiological processes.

POTASSIUM CYANIDE.

In strong KCN solutions, m/10 to m/250, a normal response is given at first, lasting from less than a minute to 30 minutes, and this is followed by a constant glow lasting for 7 minutes in m/10 and for 35 minutes in m/250 KCN (table 5). The normal response at the start is probably due to the delay in the KCN taking effect. In a concentration of m/500 the normal response lasts for several hours,

TABLE 5.—*Effect of potassium cyanide.*

Solution in sea-water.	Luminous response.[1]
m/10 (precipitate forms)	Normal response ¾ minute, then constant glow 7 minutes.
m/60	Normal response 4½ minutes, then constant glow 15 minutes.
m/125	Normal response 10 minutes, then constant glow 35 minutes.
m/250	Normal response 30 minutes, then constant glow 35 minutes.
m/500 to m/2000	Normal response >1½ hours, <12 hours.
m/4000 to m/16000	Normal response >6 days (experiment discontinued).

[1]The normal response at the start is doubtless due to the delay in taking effect.

and in solutions weaker than m/2000 there is no effect on luminescence. It is of interest to note the slight effect of KCN on oxidations connected with luminescence in comparison with its marked inhibiting effect on oxidations by plant oxidases and on the oxygen consumption of egg-cells and other animal tissues (Loeb and Wasteneys, 1913). The oxidations connected with light-production are apparently entirely different from those concerned with cell-respiration.

ABSENCE OF OXYGEN.

The effect of the lack of oxygen on *Noctiluca* was tested by subjecting the animals to an atmosphere of bubbling well-washed hydrogen from a Kipp generator. When thus treated, the luminescence gradually grows fainter for an hour until it is very faint. If air is now admitted to the tube, the animals respond on irritation as normally and just as brilliantly. Animals caught by the surface film of water on the sides of the tube give light when oxygen is admitted, even without mechanical stimulation. The mere admission of oxygen after its absence may therefore serve as a stimulus.

If the animals are kept for 2 hours, however, in the bubbling hydrogen, the luminescence goes out entirely and does not return on admitting air to the tube, the animals having probably been killed by the treatment. In a control experiment, hydrogen was bubbled through a tube containing the animals, in presence of oxygen; the animals were normal after 1 hour, but gave no response after 2 hours. It is therefore the mechanical disturbance and not the lack of oxygen which is fatal to the animals subjected for 2 hours to the hydrogen.

Light-production in *Noctiluca* is therefore dependent upon a supply of oxygen, as is to be expected. Since the cells deprived of oxygen immediately give light on admitting oxygen, even without stimulation, they must be permeable to oxygen at any time, and not merely upon stimulation and death, when light-production ordinarily takes place. That oxygen is necessary for light-production in *Noctiluca* is contrary to the ideas of Quatrefages (1850), and brings this animal in line with all the other light-producing animals in which the question has been carefully investigated.

TEMPERATURE.

With increase of temperature up to 42° or 43° C., noctilucas give a normal response. From this point to 48° or 49°, a steady glow is given and then the light goes out completely and there is no recovery if cooled immediately. With decrease in temperature, the animals flash more than normally until the temperature reaches 5° to 0°, when they give a constant glow. If kept only a few minutes at 0°, they will recover on warming and again give a normal response; but if kept at 0° for 15 minutes they do not recover.

CENTRIFUGING.

On centrifuging noctilucas for 3 to 5 minutes with a hand centrifuge, the animals form a layer at the surface of the liquid, and in each animal the protoplasm is thrown to the bottom of the cell as the heaviest material. The animals do not orient themselves in any way in the centrifuge, but the protoplasm is thrown to whatever part of the cell happens to be toward the centrifugal pole. After about an hour, many of the animals recover, and the protoplasm may be seen under the microscope returning to its normal position near the mouth-groove and flowing along protoplasmic strands to the periphery of the cell. No separation of the materials of the protoplasm into layers was observed with the centrifugal force used. The effect of centrifuging upon the luminescence is practically *nil*, for the animals give a normal response as soon as taken from the centrifuge tubes and placed in a dish of sea-water.

ELECTRICITY.

When a constant current is passed through a mass of noctilucas, the animals flash brightly at the make, continue glowing during the passage of the current, and cease to glow at the break, giving no flash, but sometimes they stay glowing after the break, and in this case the stronger the current the longer the glow lasts. If stimulated mechanically while the current is passing, they respond by a flash, just as when no current is passing.

The light comes from all parts of the noctiluca and is not restricted to anode or cathode regions. No increase in luminosity could be observed on the cathode side nor decrease on the anode side of the animal comparable with the polar effects of the current on muscle.

The tentacle movement is also influenced by the passage of a constant current. At the make, the tentacle coils up rather tightly, like a watch-spring, and at the break it uncoils, the process being repeated for a number of makes and breaks. This tentacle response is similar to the abnormal behavior of the sartorius muscle of a frog, which sometimes contracts on the make, stays contracted while the current is passing, and relaxes on the break. Spaeth (1916) similarly found that the pigment cells of fish-scales contract on the make, stay contracted during the passage, and relax on the break.

When a mass of noctilucas is subjected to the induced currents of an induction coil they respond with a flash at the break, and at the make also, if the current is strong enough.

If subjected to an interrupted induced current for 45 seconds, the animals flash on the first shock and then remain glowing, but the luminosity becomes gradually fainter. If the current is now stopped for a moment and then passed again, there is again a bright glow. The animals therefore fatigue rather readily when stimulated electrically, as they also do with mechanical stimulation.

RESPONSE OF INJURED CELLS.

If a number of noctilucas are punctured with a needle, causing the cells to collapse, and are then subjected to an interrupted current, they respond just as uninjured cells do. Such punctured and collapsed cells likewise give a normal response when stimulated mechanically—*i. e.*, by pressure of a cover-slip.

Noctilucas are injured by a few shocks of the strong induced current; the protoplasm shrinks away from the cell-wall, leaving a clear area at the periphery. If the animal is further subjected to an induced current, there is again a response, the protoplasmic area becoming luminous, while the clear peripheral area does not. After a short time the cell collapses and the animal appears as a shrunken mass of protoplasm within an irregular membrane. These shrunken animals also respond at the break of a weak induced current with a flash, just as normal cells do; and they likewise give a normal response to mechanical stimulation—*i. e.*, if pressed by a needle. It is thus shown that injury to a noctiluca does not interfere with its response to mechanical or electrical stimulation.

If, however, the injury to the cells is too great, and the cells are completely broken to pieces, they do not respond to stimulation. By pressing a mass of noctilucas through cheese-cloth, a filtrate was obtained containing many empty membranes and fragments of cells, visible under the microscope. This filtrate, although luminous, did not respond to electrical stimulation. Another filtrate obtained by pressing a mass of noctilucas through fine-meshed bolting-cloth, and tested while still luminous, did not respond to electrical stimulation. It is possible, however, that the fact that the noctiluca juice is acid may have some effect on the response in these cases.

LOCATION OF LUMINOUS MATERIAL.

When a noctiluca is giving a bright, constant glow—for instance, when treated with n/2000 to n/4000 HCl—it is fairly easy to observe the light under a microscope in a dark room. The chief luminescence comes from the main mass of protoplasm near the mouth-groove and around the periphery of the cell, while the area between these two regions is much fainter. The luminescence is a general glow over the cell, and a similar glow is suffused momentarily over the cell when a noctiluca is stimulated by a needle. This effect is, however, probably due to a close aggregation of small luminous particles, for when noctilucas are crushed under the microscope and the particles separated by means of a cover-slip, numerous points of light may be observed, similar to stars in the sky. The luminescence, therefore, apparently comes from small granules in the protoplasm, which may be freed from the cell by crushing. That the luminous granules are located in the protoplasm and not elsewhere was shown by stimulating cells injured

by electricity. As mentioned previously, a few shocks of an induced current causes the protoplasm of a noctiluca to shrink away from the membrane into a compact irregular mass; when again stimulated, the irregular mass of protoplasm gives light, while the clear peripheral area remains entirely dark. Also, in centrifuged noctilucas there is a gradual motion of luminescence in a cell corresponding roughly with the motion of the protoplasm as it resumes its normal position. These granules in the protoplasm to which the luminescence may be traced do not stain with neutral red nor with methylene blue; there are other granules, however, especially around the periphery of the cell, which do stain with neutral red.

It has not been possible to obtain two substances concerned with light-production in *Noctiluca*, as in *Pholas* (Dubois, 1913, 1914), firefly and *Cypridina* (E. N. Harvey, 1917), etc., one destroyed by heat (photogenin) and the other thermostable (photophelein), notwithstanding repeated efforts to demonstrate them. Only one substance responsible for light-production in *Noctiluca* can be demonstrated, and this substance occurs as granules and burns until it is all used up as soon as it is brought into contact with atmospheric oxygen by crushing the cells; this we may conveniently call photogenin.

ANESTHETICS.

Anesthesia may be defined as a temporary inhibition of some physiological activity and includes a wide range of processes such as cell-division, responses to stimuli of various kinds, light-production, etc. Its chief characteristic is that the condition is reversible—that is, that normal processes are resumed on removal of the anesthetic. The anesthesia may be caused by various means—the constant electric current, change of temperature, and many chemical substances. Among the latter are the salts of Mg, Ca, and other metals, as well as the more common lipoid-soluble anesthetics—chloroform, ether, alcohol, etc. This study deals with the anesthesia of light-production in *Noctiluca* by means of the lipoid-soluble anesthetics.

The only previous work showing the definite anesthesia of light-production in animals is that of E. N. Harvey (1915) on luminous bacteria; he found that the light could be inhibited completely by various alcohols and returned on dilution with sea-water.

Of the various theories of anesthesia, that of Overton (1901) and Meyer (1899) has received widest acceptance. According to this theory, anesthesia is due to lipoid solubility, the anesthetic dissolving in the lipoids of the cell. It has been shown by Overton that there is a direct ratio between the narcotizing power of anesthetics and their lipoid solubility—that is, the more soluble the narcotic in fat, the more narcotic power it has and the smaller the amount necessary for anesthetizing. Just how the solution of the anesthetic in lipoids should

change the irritability is not explained by the theory, nor does it take into account other anesthetics which are not lipoid soluble—*e. g.*, neutral salts. No distinction is made between the action of anesthetics in the membrane and in the cell interior. Traube (1913 and 1915) considers anesthesia as due to adsorption of the narcotic by surfaces in the cell, especially colloidal particles, due to the lowering of the surface tension of water. This causes a decrease in catalytic activity of the surface layer, leading to a decrease in the chemical activity of the cell, especially of oxidations. It has been shown that many substances with the same surface tension have the same physiological action, although this is not always true. The fact that dried micro-organisms free of lipoids can be anesthetized supports this theory rather than the lipoid theory (Warburg and Wiesel, 1912). On the other hand, the effect of temperature on anesthesia corresponds more closely with the effect on lipoid solubility than on adsorption of the anesthetic (Meyer, 1901).

That the narcotic acts by interfering with oxidation is held by others besides Traube. Verworn (1913) considers narcosis as an asphyxiation, since narcotized cells behave similarly to those deprived of oxygen. Mathews (1914) explains the relation of oxidation and anesthesia by assuming that protoplasm contains an unstable compound with oxygen, which breaks down on stimulation, and the anesthetic forms a stable compound with oxygen and thus prevents its liberation on stimulation. According to Mansfeld (1909), the anesthetic prevents the oxygen from entering the cell by decreasing the permeability of the surface for oxygen. In favor of these theories is the fact that oxygen consumption by certain cells and in certain oxidations has been shown to be decreased by anesthetics (Warburg, 1911). However, in these cases the concentration necessary to decrease oxidations is much greater than that necessary to produce anesthesia. Moreover, the experiments of Loeb and Wasteneys (1913) on sea-urchin eggs have shown that an anesthetic in the concentration sufficient to prevent cleavage had very little effect on oxidations. A slight decrease in temperature has a much greater effect on the rate of oxidations than an anesthetic of effective strength.

The membrane theory of Lillie (1909–1916) and Höber (1907) is also important. According to this the anesthetic affects the interior of the cell indirectly, the primary effect being upon the permeability of the cell membrane. The change in permeability is supposed to be connected with a change in the aggregation of colloidal particles, protein and lipoid. Lillie's experiments on *Arenicola* larvæ show that the narcotic prevents an increase in permeability, or in some cases causes an actual decrease. Osterhout (1913) has shown also, in *Laminaria*, that anesthetics cause a decrease in permeability, as indicated by an increase in resistance to the electric current. This theory takes into consideration the non-lipoid-soluble as well as the lipoid-soluble anesthetics.

All of these theories fall into two groups: those which consider the action of the anesthetic to be upon the cell-membrane and those which consider the action to be directly upon the cell-contents. Experiments on the anesthesia of light-production in *Noctiluca* have been carried out in the attempt to find out which of these two alternatives is correct.

Although the anesthesia of some processes has been shown to be independent of the consumption of oxygen—for instance, the cleavage of sea-urchin eggs (Loeb and Wasteneys, 1913, and Warburg, 1910)—it would seem probable that the anesthesia of light-production is dependent on oxygen. That oxygen is necessary for light-production in *Noctiluca* has been shown by keeping the cells in an oxygen-free atmosphere, in which case the luminescence stops, but reappears on admitting oxygen. The membrane is freely permeable to oxygen at any time, since those cells deprived of oxygen immediately glow on admission of oxygen. It may be noted in passing that this oxidation is different from that of other cells, as shown by the fact that KCN in relatively high concentrations has no effect, whereas it so quickly affects other oxidations.

The anesthesia must in some way affect the oxidation which is concerned in light-production. Does it do so by preventing oxygen from entering the cell, or does it prevent the utilization of the oxygen already present in the cell? In other words, does the anesthetic act upon the membrane or directly upon the cell interior?

In my experiments various lipoid-soluble anesthetics were tried and the effect of the anesthetic has been in all cases not to prevent light-production altogether, but to prevent a normal response—*i. e.*, a flashing on stimulation. In all the effective concentrations, the animals under the anesthetic produce a steady glow, so faint in some cases that it is not noticeable unless the animals are present in large number. When returned to sea-water, if not left too long in the solution, the steady glow ceases and the normal response returns; this is therefore a reversible phenomenon and a true case of anesthesia. The best concentrations for anesthetizing, as seen by referring to table 6, were: 1/3 saturated chloroform, where the steady glow lasted 2 hours; m/8 ether and m/8 butyl alcohol, steady glow lasting $1\frac{1}{2}$ hours; 1/16 saturated thymol, lasting 1 hour; methyl alcohol, lasting 30 minutes, and 1/4 to 1/8 chloretone, lasting 15 minutes. If returned to sea-water after the period of steady glow, the animals gave no response, the prolonged anesthesia causing death. The tentacle motion was also stopped by the anesthetic in the same concentrations as prevented the normal light-response. The effect on the tentacle was, however, much slower than the effect on light-production, but took place during the early part of the period of constant glow.

The question as to whether the effect of the anesthetic is upon the interior of the cell or upon the cell membrane was tested by destroying membranes, thus allowing oxygen to enter. A number of noctilucas were

subjected to m/8 butyl alcohol for an hour in a tube containing sand. Some were then returned to sea-water and recovered, showing that the animals were truly narcotized. The remaining animals were shaken up and broken to pieces by the sand. At the same time, a control lot kept for the same period in sea-water in a tube containing sand were shaken and broken up. A comparison of the two tubes showed that the anesthetized animals were not nearly so bright as the control lot. A microscopic examination of the material showed that the cells were completely broken to pieces into irregular fragments by the sand. A

TABLE 6.—*Effect of anesthetics.*

Solution in sea-water.	Luminous response.	Duration of tentacle movement.
Chloroform:		
Saturated	Constant glow 1 to 2 minutes.	
1/2 saturated	Constant glow 4 to 8 minutes.	
1/3 saturated	Constant glow 2 hours, then normal response, owing to evaporation of chloroform.	30 minutes.
1/4 to 1/8 saturated	Normal response >12 hours (exp. discontinued).	
Ether:		
m/2	Constant glow 4 minutes	<5 minutes.
m/4	Constant glow 30 minutes; becomes brighter, then fainter.	<5 minutes.
m/8	Constant glow 90 minutes	<30 minutes.
m/16	Normal response >1 day (exp. discontinued).	
m		
Ethyl alcohol:		
2m	Constant glow 1 minute	
1.5m	Constant glow 5 minutes	<5 minutes.
m	Constant glow 30 minutes	<10 minutes.
m/2 to m/4	Normal response >14 hours (exp. discontinued).	
Butyl alcohol:		
m/2		
m/4	Constant glow 1 minute	
m/8	Constant glow 5 minutes	
m/16 to m/32	Constant glow 1½ hours (faint)	<20 minutes.
	Normal response >14 hours (exp. discontinued).	
Chloretone:		
Saturated	No response	
1/2 saturated	Constant glow 4 minutes	Stops immediately
1/4 to 1/8 saturated	Constant glow 15 minutes (faint)	<10 minutes.
1/16 to 1/32 saturated	Normal response >24 hours (exp. discontinued).	
Thymol:		
Saturated to 1/4 saturated.	Constant glow 1½ to 2 minutes	
1/8 saturated	Constant glow 6 minutes	
1/16 saturated	Constant glow <1 hour, normal response 4 minutes at start (poor).[1]	<15 minutes
1/32 saturated	Normal response <18 hours (poor)	
1/64 to 1/128 saturated.	Normal response >5 days (exp. discontinued.	

[1]The normal response at the start is doubtless due to the delay in taking effect on certain of the animals,

similar experiment was tried with animals subjected to m/8 ether for 10 minutes with the same result. In these experiments the cell-membranes were destroyed, allowing oxygen to enter the cell directly, and the effect was different for the anesthetized cells and the control. The anesthetic must therefore act, not on the cell-membrane by preventing oxygen from entering the cell, but directly upon the cell interior, probably attacking the mechanism of the utilization of oxygen in the cell.

A criticism of the foregoing experiments might be offered—that, owing to the length of time that the cells were subjected to the anesthetic, substances present in the cells might be used up and the cells be fatigued. The following experiment showed that such was not the case: Three lots of animals were put into tubes with sand, two immersed in m/8 ether and the third in sea-water. One of the ether tubes was very slightly agitated almost immediately and did not increase in brightness as normal animals do on slight agitation, showing that the animals were narcotized. The other ether tube was shaken vigorously at once so as to break up the animals, and it became slightly brighter. The control tube with sea-water, when also shaken vigorously at the same time, became markedly brighter, showing a striking contrast to the other two tubes. In this experiment there was not sufficient time for any substances in the cells to be used up, and yet the narcotized cells gave a much weaker light than the control lot on admission of oxygen by destroying the membranes. The evidence from these experiments on *Noctiluca* would therefore argue against the membrane theory of anesthesia, since the anesthesia of light-production takes place independently of the cell-membrane; the effect of the anesthetic seems to be upon the mechanism of the oxidation process inside the cell.

SUMMARY.

1. The specific gravity of *Noctiluca* is less than that of sea-water, so that normally the animals float at the surface. Since they contain no air-bubbles or large oil-drops, their lower specific gravity must be due to a lower salt-content than that of sea-water. When placed in a mixture of 4 sea-water to 6 fresh-water, the animals sink, their salt-content being now greater than that of the surrounding medium, but later they rise to the surface, a process independent of the movement of the tentacle. They therefore absorb water until their salt-content is again less than the medium, thus reestablishing their normal relation to the surrounding medium. This water must be absorbed against the osmotic pressure of the salts of sea-water, a process contrary to physical laws. The animals can not only lessen their specific gravity, but can also increase it, as shown by the fact that they sink to the bottom of the sea on windy days. Anesthetics, acids and alkalies, KCN, and the pure salts of sea-water do not interfere with this regu-

latory mechanism, except when they cause irreversible changes and death; dead noctilucas always sink to the bottom.

2. Light-production in *Noctiluca* normally occurs only on stimulation of any kind, and is a momentary bright flash. The only other light-response is a steady glow occurring before death and under many experimental conditions—*e. g.*, diluting with fresh-water, addition of acids and alkalies, KCN, cold (5° to 0° C.), heat (43° to 49° C.), anesthetics, and a constant galvanic electric current.

3. In very dilute sea-water (3 sea-water to 7 fresh-water down to pure fresh-water) the animals give a constant glow for a few minutes and then die. If the sea-water is diluted with isotonic cane-sugar instead of water, a normal response is given even with 1 sea-water to 9 cane-sugar. But in isotonic cane-sugar alone a normal response is given for only a short period, followed by a steady glow and death, showing that the animals must be bathed by a medium of minimal salt-content as well as of definite osmotic pressure.

4. The normal light-response of *Noctiluca* is dependent on the balanced salts of sea-water. Isotonic Na, K, Ca, and Mg are all toxic if alone in solution.

5. n/2000 HCl and n/4000 benzoic acid in neutral artificial sea-water cause a steady glow for 20 minutes; n/2000 NH_4OH and n/500 NaOH give a steady glow for 6 and 30 minutes respectively. In NaOH the luminescence stops *before* the penetration of the alkali, and in NH_4OH *after* the penetration, as indicated by the color-change of animals stained in neutral red.

6. KCN has little effect on light-production in relatively strong concentrations. Animals in n/250 KCN respond normally for 30 minutes and then give a steady glow for 35 minutes.

7. Oxygen is necessary for light-production. Animals deprived of oxygen immediately glow on the admission of oxygen even without stimulation.

8. When the temperature is gradually raised, a steady glow appears at 42° C. and stops at 48°, when the animals die. When the temperature is lowered a steady glow appears at 5° and continues to 0°. The animals will recover on warming if kept for only a few minutes at 0°.

9. Centrifuging has no effect on the luminous response, although the protoplasm is thrown to whatever part of the cell happens to be away from the axis of the centrifuge. On standing, the protoplasm returns to its normal position under the tentacle.

10. With a constant galvanic current noctilucas flash at the make, stay glowing during the passage of the current, and stop glowing at the break with no flash. Animals will respond normally to mechanical stimulation during the passage of the current. The luminescence is uniform throughout the organisms, no polar effects being observed. Induced shocks call forth the usual flash caused by stimulation. The animals fatigue readily with interrupted induced shocks.

11. Noctilucas injured by puncturing with a needle or by a strong induced current respond to mechanical or electrical stimulation. If completely broken to pieces by pressing through cheese-cloth, the filtrate gives a constant glow, but there is no flashing on stimulation.

12. The luminescence is traceable to points of light coming from granules (photogenin) in the protoplasm. No photophelein could be demonstrated.

13. Noctilucas may be anesthetized by m/8 ether, 1/3 saturated chloroform, 1/16 saturated thymol, 1/8 saturated chloretone, m ethyl and m/8 butyl alcohol, so that they fail to give a flash on stimulation, but they always give a very faint glow; this disappears and the normal response returns on removing the anesthetic. Light-production is dependent on the consumption of oxygen. Whether the anesthesia of luminous cells is due to the fact that oxygen can not pass through the membrane, or to the fact that it can not be used, was tested by an experiment in which the cell-substance of anesthetized cells was permitted to come into contact with dissolved oxygen. Narcotized cells were broken up by shaking with sand and it was found that they produced only a faint light, whereas normal cells so treated became very brilliant. The anesthetic must therefore attack the mechanism of the utilization of oxygen in the cell, and not the permeability of the cell-membrane for oxygen.

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X.

POLYCHÆTOUS ANNELIDS FROM FLORIDA, PORTO RICO, BERMUDA, AND THE BAHAMAS.

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Three plates.

POLYCHÆTOUS ANNELIDS FROM FLORIDA, PORTO RICO, BERMUDA, AND THE BAHAMAS.

By Aaron L. Treadwell.

INTRODUCTION.

In 1911 the present author published a paper entitled "Polychætous Annelids from the Dry Tortugas, Florida."[1] This was the first of a proposed series of systematic papers on the polychætous annelids of the West Indian region, collected under the auspices of the Department of Marine Biology of the Carnegie Institution of Washington, my main object having been the preparation of a monograph of the West Indian Leodicidæ. It has seemed best, since the final publication must be deferred, to publish a preliminary description of some new species belonging to this family, as well as new species of other families which have been collected incidentally in this work. I have included also a description of a new sabellid belonging to the collection of the American Museum of Natural History.

FAMILY SYLLIDÆ.

Odontosyllis octodentata n. sp.

A relatively large species, approximately 40 mm. long and 2.5 mm. in width. The prostomium (plate 1, fig. 1) is broader than long, with the anterior margin broader than the posterior, the lateral angles rounded. The posterior margin has a slight depression which is not shown in the figure because a lobe from the anterior margin of the peristomium extends over it. The palps are separate from one another, and prominent, not sharply separated from the prostomium, so that they seem almost to be forward prolongations of it. Eyes 2 pairs, brown, anterior pair the larger and farther apart than posterior pair. The median tentacle was noticeably more slender than the lateral and none of these extends much beyond the tip of the palp. None of the tentacles were well preserved and I am uncertain as to their normal appearance.

Pharynx short, with ventrally a row of 8 subequal teeth. The peristomium is prolonged dorsally into a broad lobe which covers the posterior surface of the prostomium. Tentacular cirri are slender, the dorsal ones being the largest. Dorsal cirri of later somites similar in form but successively smaller.

Parapodium (plate 1, fig. 2), with its end prolonged into equal anterior and posterior lobes and with an acicula with its apex coming to the surface just dorsal to these. Ventral cirrus ovate in outline, shorter than setigerous lobe. Dorsal cirrus long and slender. The setæ (fig. 3) with a very long basal joint, the longest reaching nearly halfway to the end of the dorsal cirrus. Terminal joint with a very short terminal and a single subapical tooth. Apex of basal portion with minute denticulations.

Type in American Museum of Natural History.

Collected at the Dry Tortugas in 1914.

[1]Bulletin of the American Museum of Natural History, vol. 30.

FAMILY POLYNOIDÆ.

Lepidonotus inquilinus n. sp.

Length of type 14 mm.; greatest breadth at end of anterior third 4 mm.; body tapering from here in both directions, but the head is broader than the pygidium.

The head (plate 1, fig. 4) is oval with the transverse diameter the longer, with a narrow median posterior depression and a much wider anterior one, the latter filled with the base of the median tentacle. Posterior eyes very small and situated on the dorso-posterior surface, the anterior ones larger, on the antero-ventral surface, and not visible from above. At the anterior margin the head is continued into the bases of the lateral tentacles, with a constriction at the base of the latter. Lateral tentacle not more than half as long as head, lanceolate, with acute tip. The other tentacles and tentacular cirri had been lost. Palps about 6 times the length of the head, perfectly smooth.

The body has 39 somites and 15 pairs of elytra. In alcohol the color is a yellowish brown with no trace of pigmentation except on the elytra. On each elytron a dark-brown pigment patch extends from the attachment of the elytrophore toward the median longitudinal line of the body. This is crossed by a definite white line at the position of the inner margin of the elytrophore and does not reach the edge of the elytron. (Plate 1, fig. 5.)

First parapodium (plate 1, fig. 6) with prominent antero-dorsal lip on the neuropodium, the notopodium slender and cirrus-like, with an acicula and a bundle of delicate, curved, minutely denticulate setæ. The ventral cirrus is very slender, reaching nearly to the tip of the neuropodium. A parapodium from the middle of the body (plate 1, fig. 7) has the neuropodia and notopodia much as in the anterior one, but the antero-dorsal lobe of the notopodium is more acutely conical in form. The dorsal cirrus has a stout basal joint, its terminal joint slender and tapering, extending beyond the apex of the parapodium. The ventral cirrus is situated on the neuropodium about one-third of its length from the apex. Each lobe of the parapodium has an aciculum and the arrangement of setæ is much as in the earlier somite.

In the middle parapodium are three kinds of setæ:

(1) Notopodial: these are all alike, each seta being very long and slender, curving gently toward apex and with very delicate teeth along one edge; fine lines across the seta possibly indicate minute plates of which the teeth are the free ends (plate 1, fig. 8).

(2) Dorsal neuropodial: with long slender shafts, but with the terminal third of the seta elongated lanceolate in form; minute transverse plates in this terminal third protrude from the margin so as to give it a minutely toothed appearance (plate 1, fig. 9).

(3) Ventral neuropodial: those lying next to the dorsal ones are much like them in length, but have heavier shafts and the terminal swelling is shorter, so that the lanceolate end is less elongated; the more ventrally placed ones are shorter, so that those on the very ventral side are not more than half as long as the dorsal ones; each (plate 1, fig. 10) has 5 or more rows of finely toothed plates beyond the base of the lanceolate portion, with very fine transverse plates between the last of these and the apex of the seta. The apex in both kinds of ventral setæ is bifurcate.

Collected July 6, 1915, on a *Marphysa* from Mangrove Key, in Key West Harbor.

Type in American Museum of Natural History.

Lepidasthenia varius n. sp.

Body variable in number of somites and length; one entire individual of 50 somites measured 30 mm., while an incomplete specimen contained 80 somites and measured 48 mm. Greatest width of larger specimen 3 mm.

The head (plate 1, fig. 11) with sides smoothly rounded laterally and tapering gently to the bases of the tentacles. The posterior margin is deeply incised and a faint groove is carried over the dorsal surface to the base of the median tentacle. The greatest width is behind the middle, and this is about equal to the length. There are apparently 2 pairs of eyes, the anterior larger and situated on the antero-lateral surface, the posterior near the posterior margin of the head. Neither eye showed any pigment, their position being indicated by an elevated patch of whitish epithelium.

The tentacles are carried on prominent basal joints, the median one being larger than either of the others. The median and one lateral were absent in both specimens. The remaining lateral was about $2\frac{1}{2}$ times as long as the head, quite uniform in diameter except for a subterminal swelling and acute apex. Tentacular cirri similar in form to the tentacles, but larger and with traces of pigmentation near their ends. The palps are much longer than the cirri, tapering very gradually until near the end, then abruptly narrowing to an acute apex. With high power a few very small papillæ can be seen near the apex. The ventral cirrus of the second somite (first setigerous) and the dorsal cirri of the next somite much like the tentacles in form, but shorter and with less evident subterminal swellings. Later dorsal cirri are successively smaller and throughout the greater part of the body are conical, with acute tip. Of the ventral cirri, all but the first pair are small and conical; the anal cirri are much like the dorsal in form.

Elytra are carried on somites 2, 4, 5, etc., to 23, 27, and behind this two somites are regularly interposed between each pair of elytron-bearing somites. The first pair covers the head, while succeeding ones are smaller (*cf.* fig. 12, plate 1, of the first, with fig. 13, plate 1, of the eighth) and because of their small size and transparency they are easily overlooked and may seem to be absent. Toward the posterior end they are larger and cover more of the body, the terminal somites being entirely covered. They are all transparent, but behind the middle of the body each carries a patch of pigment, surrounding the point of attachment of the elytrophore and extending to the inner margin of the elytron. There is a very distinct line between this pigment and the point of attachment, giving the body in this region an ocellated appearance. The elytra all have smooth margins and are oval or round in outline.

The first parapodium (plate 1, fig. 12) cylindrical, very slightly expanded at apex, with presetal and postsetal lobes, the latter much the larger and curved upward at end. Between the two is a single row of stout setæ, their apices extending for only a short distance beyond the postsetal lobe. There is no indication of dorsal setæ, but two aciculæ extend, one into the dorsal part of the parapodium and one into the ventral.

The elytrophore is expanded at the apex, carrying the large rounded, nearly oval, elytron. The ventral cirrus is on a prominent cirrophore and is nearly 3 times as long as the parapodium, abruptly narrowing toward the end to form a slender acute tip.

The second parapodium is very similar to the first in appearance and arrangement of setæ (plate 1, fig. 14), with a dorsal cirrus resembling the ventral cirrus of the first parapodium. The ventral cirrus has a terminal joint in the form of a narrow cone, about as long as the parapodium. The fifteenth parapodium (plate 1, fig. 13) with very small achætous notopodium and a neuropodium much like those of earlier somites, but with a very small elytron. The

ventral cirrus of the fifteenth is similar to that of the second. There is a single row of very stout setæ and there are aciculæ in both noto- and neuropodia. This general form of parapodium is continued to the end of the body, though the anterior and posterior lips of the parapodia become more nearly equal, so that the organ when viewed from above has a bifurcated appearance. The nephridial papillæ are cylindrical and very prominent.

The setæ are essentially alike throughout the body, differing only in the relative size of the different parts. Each has a stout shaft (plate 1, figs. 15 and 16), widening toward the end but narrowing to a pointed apex and with a sharp subterminal tooth. On their dorsally directed surfaces each has a series of alternately arranged plates with their free ends finely toothed. The setæ of the dorsal part of the setæ bundle have more numerous plates than ventrally, the middle ones being intermediate as regards this character.

One of the two specimens showed an indication of a brown color over the head and bases of the antennæ. Somites 2 and 3 were uncolored. From somite 5 to somite 20 the dorsum has a brown band, due to transverse markings in each somite; these are shorter than the somite diameter, so that the margins of the somites above the parapodia appear uncolored, but somites 6, 8, 12, 14, and 20 are uncolored, thus forming interruptions in this band. Behind somite 20 the markings occur only in the elytra-bearing somites.

Collected in the Dry Tortugas in 1914.

Type and cotype in the American Museum of Natural History.

Harmothoe variegata n. sp.

The type specimen was collected at the Dry Tortugas in 1909. I also have specimens collected at St. Augustine, Florida, and loaned to me by Professor Verrill.

The type is 26 mm. long and 5 mm. wide in its widest part, near the middle; from here it tapers toward both ends, but more noticeably so posteriorly than anteriorly. There are 39 somites, and 15 pairs of elytra.

The most striking part of the coloration of the alcoholic specimens is formed by the marking of the elytra. The inner and posterior margin of each elytron is marked with a dark line, which in the inner posterior quadrant is continued as a pigment patch, of a slightly lighter shade than the margin, to reach the elytrophore attachment (plate 1, fig. 17). When the elytra are in place the general effect is that of a broad median dark band on each elytron, the lateral posterior quadrant of each lighter-colored, but surrounded by a narrow marginal band. The remainder of the dorsal surface is uncolored, except that at the beginning of the posterior quadrant prominent black spots appear on the base and apex of some parapodia, usually only on elytra-bearing somites. Approximately the last 10 somites have a median dorsal pigment patch and 6 are uncovered by elytra.

Ventrally the margins of the mouth are pigmented, the pigmentation continuing on either side as a single patch at the base of each parapodium, toward the posterior end extending so as to cover a large part of the ventral surface.

The head is only slightly rounded, a little longer than broad, the anterior notch very narrow (plate 1, fig. 18). Peaks of head very slightly marked off from the ceratophores of the lateral tentacles, the latter about one-quarter as long as the head and densely pigmented. The terminal joint of the tentacle is slender, its entire length not more than twice that of the ceratophore, its tip filiform. The basal half of this terminal joint is pigmented, the terminal half being also colored, but not so deeply as the basal; apex uncolored. The median tentacle had been lost, but its ceratophore is large, overlapping the lateral ones, pigmented at its end. Palps rather stout, about 3 times as long

as the head, their middle third pigmented. On the type there remained one ventral tentacular cirrus, slender and extending to about the middle of the palp, with a median and terminal pigment patch; all other tentacular cirri were lost, as were most of the dorsal cirri. Those dorsal cirri remaining were slender, gently tapering to the apex, more or less pigmented for the terminal half, but with a subterminal white patch. The anal cirri are larger than the dorsal and entirely pigmented.

The elytra are very similar in character throughout the body, with an entire margin and without papillæ; they are transparent, so that although they overlap, the pigment of the under one shows through the upper and gives the impression of a continuous line.

The notopodium of the first parapodium is thicker than the neuropodium and extends about half as far from the body. It bears from the bottom of an oval terminal depression a tuft of heavy setæ, of which the ventralmost are especially large (plate 2, fig. 1). The acicula extends into a conical expansion ventral to this depression.

Neuropodium much wrinkled, its dorso-anterior angle prolonged into a conical process; it carries a single acicula and large setæ. The ventral cirrus is large, equal in size to the dorsal cirrus of the second parapodium; its elytron was broken from the specimen figured, only its ceratophore being represented.

The second parapodium has a less developed notopodium than the first, but the setæ in the two are very similar. Neuropodium more conical than in the first, with a finger-shaped process at end of antero-dorsal lobe. Ventral cirrus two-thirds as long as the neuropodium, the dorsal cirrus longer than the parapodium, tapering gradually to the apex.

Toward the posterior end (plate 2, fig. 2) of the fifteenth from the posterior end the ventral cirri become very small, while an anterior lobe appears in the notopodium. In other respects the parts of the parapodium have a relative form much as anteriorly, and the very large setæ continue in the notopodium.

The large setæ of the dorsal tuft vary much in size, but the ventral ones are largest. They have a striated axis, which terminates in a blunt point and show traces of rows of minute teeth along the shaft. The apices were invariably covered with a thick brownish incrustation, which made it impossible to determine their exact form. The setæ of the ventral bundle (plate 2, fig. 3) with long tapering shaft and a subterminal tooth at some distance below the apex. The expanded portion of the seta with numerous rows of very minute teeth, apparently extending entirely around it, but larger on the convex surface; these stop at the subterminal tooth.

Type in American Museum of Natural History. Cotype in the Yale University Museum.

Pontogenia maggiæ Augener.

Pontogenia maggiæ Augener, 1906, p. 103.

A single specimen, dredged July 19, 1915, south of Loggerhead Key, agrees closely with Augener's description, though if his figure of the head is accurately drawn his specimen was poorly preserved. While there are lateral lobes as he figures, they are not as deeply incised as is shown in his figure and they are carried on the dorso-lateral surface, so that the head is broadest at its junction with somite 1. The median tentacle is shorter than the dorsal cirrus and very delicate. Each eye-stalk carries dorsally a small eye, and antero-ventrally a much larger one, somewhat as in Ehlers's *Pontogenia sericoma* (Ehlers, 1887, plate 7, fig. 2), but unlike that species these are carried on prominent stalks. Brown papillæ very abundant over the entire ventral surface,

but occurring also on the head and dorsal surfaces of the elytræ, form a noticeable feature of this species.

Each somite with one or two very heavy, long, glassy setæ, longer than diameter of body and lying across its dorsal surface. If I understand correctly Augener's description, these are distinct from his "starke dorsale borste," which is shorter than these. I do not find in the latter any minute tubercles and would suggest that perhaps what he saw were particles of foreign matter.

FAMILY PHYLLODOCIDÆ.

Phyllodoce tortugæ n. sp.

Body of type 120 mm. long, of more than 200 somites. Prostomium 1.5 mm. in diameter, rather longer than broad (plate 2, fig. 4). Anterior and lateral margins of prostomium rounded, but posterior half of lateral margin nearly straight. Posterior margin deeply incised, and a nuchal papilla projects into this incision. Four subequal tentacles having rounded ends, their length not more than one-fourth that of head. Eyes one pair, large, black.

The ceratophores of all tentacular cirri are prominent, those of the dorsal ones on somite 2 being the longest. The terminal joint of the first cirrus and the ventral one of the second pair are very similar in form and size, while the dorsal one on somite 2 is the longest, reaching to the ninth somite; cirrus of third somite intermediate in length between the other two.

The partially protuded proboscis bears on either side a dense arrangement of disk-like papillæ, a median dorsal and ventral region being free from them.

The greatest width of the body, about 3 mm., was reached at somite 18. The anal cirri were much distorted in the type. On a cotype which was regenerating the posterior end, there was a single anal cirrus similar in form to the tentacular cirri, but much smaller.

Notocirri of anterior region obovate and inconspicuous in preserved material, standing well out from surface of body; they are larger and more conspicuous toward the middle of the body, and decidedly overlap one another, though at no time do they cover any very large part of the surface.

The parapodia (plate 2, fig. 5, of the tenth) with a bifid anterior lip, the posterior lip shorter and rounded (shown in dotted line in figure). A single acicula extends through the middle of the parapodium, the latter bearing on the end a row of about 10 compound setæ. The notocirrus is carried on a very stout ceratophore. The neurocirrus is on the postero-ventral face of the parapodium, its lower margin broadly rounded, bending up to form an acute angle with the straight upper margin. Later parapodia are similar to this in every detail, with the possible exception of a slight increase in the size of the notocirrus.

Seta from tenth parapodium (plate 2, fig. 6) slender with the apex of the basal joint covered with spines on its convex surface; terminal joint long, slender, fine-pointed, with row of minute teeth along the concave surface. The setæ of later somites resemble these in form, but the basal joint becomes longer, causing the seta to protrude considerably beyond the parapodium.

Type and cotype in American Museum of Natural History.

The type was collected at Loggerhead Key in the Dry Tortugas in 1914. The cotype was obtained by dredging about 10 miles south of Loggerhead in 1915.

FAMILY LEODICIDÆ.

Lumbrinereis cingulata n. sp.

An unusually small representative of this genus, the largest individuals being not over 40 mm. long with a prostomial width of 1 mm. The type after preservation was 37 mm. long and had 98 somites.

The prostomium when extended is broadly rounded, its length a little greater than its breadth (plate 2, fig. 7). There are no eyes, but some individuals have two relatively rather large pigment patches near the anterior border. The head, both dorsally and ventrally, is thickly studded with minute tubercles, clearly seen only under rather high magnification. These appear dark by transmitted, white by reflected light.

The first two somites are about equal in size (with tubercles similar to those on the prostomium), and each marked dorsally by two prominent bands of pigment spots. On later somites the tubercles are present, but except on the last two somites are less numerous than anteriorly. Anteriorly the somites behind the second have a narrow transverse band of pigment spots with occasionally much fainter bands at the margins of the tuberculated areas; they disappear entirely after the thirtieth somite. Ten or more of the most posterior somites have prominent ventral pigment patches which fuse to form a line that is wider in the center of each somite than intersegmentally. The body is very soft and easily broken, and secretes large amounts of mucus when put in clean water. There are two pairs of stout, unequal anal cirri.

The parapodia are similar throughout the body. Each (plate 2, fig. 8) has an anterior and a posterior lobe, the latter much the larger. I was unable to find any aciculæ. Setæ of two kinds. In the anterior somites the most prominent are the winged variety (plate 2, fig. 9); each has two winged processes and its axis is drawn out in an acute point, often much longer than in the one figured. At the sixteenth parapod in two specimens I examined (I am not sure that this is a constant position) the winged setæ disappear and hooded forms (plate 2, fig. 10) take their place. In most cases it seemed as if only winged ones were present in anterior somites, but in the fifth parapodium of one specimen was one hooded form which barely protruded from the surface.

The maxillæ (plate 2, fig. 11) are dark in color. The base is roughly triangular, very dark brown, with lighter margins. Basal portion of forceps rather broad and long, extending fully half the length, terminal portion slender and curved. A dark-brown pigment covers the terminal portion and continues as a dark margin along the inner edge of the basal portion, the remainder of the forceps being much lighter in color. The large paired plates have each 5 large, dark-brown teeth, the remainder being colored like the lighter part of the forceps. Of the two pairs of distal paired plates, the first has 3 teeth, the second 2; each is continued laterally into a chitinous plate dotted with black. The left-hand plates in the figure have been turned over. The mandibles are very delicate and difficult to separate from the maxillæ; they are nearly transparent, the only definite pigment being a dark spot on each near its outer anterior angle. The terminal beveled plate is not divided in the middle line. (Plate 2, fig. 12)

Collected at the Tortugas, where only 3 individuals were found, living in the crevices of broken coral, which make up a coarse gravel on parts of the bottom about 12 miles south of Loggerhead Key. They were common in Bermuda, living in the crevices of the porous surface of coral rocks, just below low-water mark, and some were found in a sponge. The type is from Bermuda.

Type in the American Museum of Natural History.

Marphysa viridis n. sp.

The type specimen, preserved and much contracted, was 100 mm. long, 2.5 mm. broad at peristomium, and 5 mm. broad at point of greatest width, which was at middle of body. It had approximately 200 somites.

In life the head and first somite are of a decidedly greenish color; remainder of body colored only by the blood seen through body-wall and gills. The head is distinctly bilobed and capable of considerable changes of form (plate 2, fig. 13). The tentacles are slender, colorless, and extend to the third somite. In life the eyes are prominent, but become less so after preservation. In preserved material the body as far as the ninth somite is very iridescent, but behind this it is of a uniform grayish brown.

Peristomium about as long as head, a little wider on posterior than on anterior margin, and only faintly marked off from the second somite, which is about half as long as it. Succeeding somites practically uniform in width and length until they narrow at posterior end. Anal cirri inconspicuous, but one of the two pairs much longer than the other.

The gills begin in the type specimen on the right side on somite 25 with a double filament; on the left they begin with one filament on 24, becoming double on 25. In later somites the filaments increase rapidly in length and new ones appear irregularly, the largest number I could find on the type being 5. Toward the posterior end the number again diminishes, and the last gill was on the twenty-fourth somite from the pygidium. The branches of all gills are long and straight, but except in contracted individuals they do not meet across the dorsal surface of the body (plate 2, fig. 14).

First parapodium with dorsal and ventral cirri about equal in length and with a conical post-setal lobe. The setal lobe bears several aciculæ (plate 2, fig. 15). The tenth parapodium with a rounded dorso-lateral post-setal lobe, the setal portion with two lobes, one dorso-posterior, the other ventro-anterior. The dorsal cirrus has a constricted base, then broadens, and finally narrows to a rounded tip. The ventral cirrus has a broad base of attachment and is bluntly rounded at apex.

A parapodium from the middle of the body has rounded setal lobe, whose anterior and posterior lips are about equal in size (plate 2, fig. 14). The dorsal cirrus is small and does not extend much beyond the setal lobe. The ventral cirrus has a broad base, and a small cylindrical terminal portion. The gill filaments are about equal in length and arise from a broad base. There are two dorsal aciculæ having rounded ends, while a ventral acicula with bifid end emerges just dorsal to the ventral cirrus. Behind the gill-bearing somites the cirri become more prominent, the dorsal being the more slender of the two.

Simple setæ slender with swollen pointed apex having entire edges. Compound seta (plate 2, fig. 16) has prominent serrations on the end of the basal joint, the terminal joint long, gently curved, without teeth. Pectinate setæ of two kinds; in the anterior somites they have about 20 very minute teeth, the end ones being a trifle longer than the others (plate 2, fig. 17). Posteriorly are found asymmetrical pectinate setæ (plate 2, fig. 18) flattened more than the others and with only about 8 prominent teeth. In the middle somites both kinds may occur. The dorsal aciculæ are rounded at the end, and are darker in anterior than in posterior somites. The ventral aciculæ are lighter in color than these, and are bifid at the end. In the posterior somites there occur, what I could not find in the anterior, needle aciculæ extending into the dorsal cirrus.

Jaws very dark, the forceps large with respect to the base, curved through 20°. The proximal paired plates have 5 teeth on the left side and 6 on the

right. Distal paired plates with 7 on the right and 4 on the left. The unpaired plate with 8 (plate 3, fig. 1) teeth. The mandibles had very long, slender, dark shafts, the beveled portion being small and its surface white, except for a black outer edge (plate 3, fig. 2).

Collected July 1915 at Mangrove Key, Key West Harbor; Boca Grande; and at Marquesas, in sandy mud within digging distance of low-water mark.

Type in American Museum of Natural History.

Marphysa nobilis n. sp.

A large species, one specimen of average size measuring, after preservation, 350 mm. in length, with a peristomial diameter of 4 mm. The three somites following the prostomium were of the same breadth as the prostomium, but there was a later gradual enlargement to 7 mm. at the seventeenth somite. Behind this there was a gradual narrowing to the posterior end, this narrowing being more evident from somite 17 to somite 30. These measurements were taken on preserved material in which the relative proportions may not have been exactly as in life.

The body of the living animal is a flesh color due to the contained blood, and anteriorly is markedly iridescent. The only pigmentation of the surface is in the form of green bands on the otherwise colorless tentacles and numerous yellowish spots scattered over the anterior surface of the body. The posterior part of the body during life is decidedly transparent. Preserved material is uniformly brownish gray. In life, the red gills give a decided tint to the middle portion of the body.

The prostomium (plate 3, fig. 3) is bilobed, though the depth of the median depression varies at different times during life. When fully expanded its anterior edge is nearly straight and it tapers on either side to a narrower base. The tentacles are longer than the prostomium, approximately equal in size, and only very gradually tapering to the apex. The green color noticeable in living material is lost on preservation, and it is then seen that a constriction occurs at each green band; this resembles an articulation, but I think is not a true jointing. There is one pair of small eyes.

The peristomium is rather short, though its absolute size naturally varies with the degree of expansion. The constriction between it and the second somite is very obscure, so that the two are practically continuous.

The gills begin as a single filament on the region of the twenty-fourth to the twenty-ninth somite and acquire a second filament a few somites behind this. They later have as many as 6 filaments (plate 3, fig. 4) and when fully developed meet from opposite sides across the dorsal surface. Posteriorly they become smaller and finally disappear entirely at about 45 somites from the posterior end.

The first parapodium (plate 3, fig. 5) with prominent dorsal and ventral cirri and a long, cirrus-shaped, posterior lobe. The anterior lobe is truncated. Two short, very black aciculæ are in this parapodium and it carries dorsally a single tuft of needle setæ. The ventral seta tuft is short and lies behind the ventral cirrus and contains compound and pectinate setæ. The tenth parapodium has a large posterior lobe with the apex bent dorsally and a truncated anterior lobe. The ventral cirrus is conical, the dorsal with a decided lobe on its ventral surface. Three aciculæ extend beyond the end of the parapodium. Parapodium 150 (plate 3, fig. 4) shows much less distinction between anterior and posterior margins than do the anterior ones. The ventral cirrus is small, borne on a rounded base, the dorsal cirrus also small with a ventral lobe. The gill is rather thick and heavy at the base, but soon divides into filaments. The setæ are as in anterior somites and there are two aciculæ.

In the body at the base of the gill is a black spot. Toward the posterior end the form of the parapodium changes very little, but the cirri become slender and a ventral light-brown acicula makes its appearance.

The simple setæ are long, with a broadened terminal portion, but without noticeable fin or denticulations. The compound setæ have very long terminal joints, not toothed at the edge (plate 3, fig. 6). This terminal joint is relatively shorter through the gill region than anteriorly. Pectinate setæ of two forms; anteriorly with 20 to 25 relatively very short teeth, the terminal ones not very long; through the middle of the body a second form appears, having not over 12 very prominent teeth (plate 3, fig. 7). These are more numerous than the other form in the region of the gills. The dorsal aciculæ are black with rounded ends, the posterior ventral ones lighter in color and with a terminal bifurcation.

The jaw apparatus is dark brown. The basal portion of the forceps is short and the median portion rather long, terminal portion gently curved, concave on inner surface. In larger individuals each inner paired plate has 4 teeth, though in immature specimens the proximal tooth may not appear. Outer paired plates with 7 teeth on the right side, 4 on the left, 2 of these being much larger than the others (plate 3, fig. 8). Unpaired plate with 6 teeth. Beyond these teeth the margin is usually smooth, but denticulations may appear in it. On either side beyond the toothed plates is a thin plate with its upper angle curved to form a tooth-like process. The mandibles have rounded shafts and are lighter in color than the maxillæ, their apical beveled portion covered with a whitish deposit. (Plate 3, fig. 9.)

Collected first at Mangrove Key in Key West Harbor in sand exposed at extreme low tide, in June 1915. It was fairly abundant in this locality and more were collected there in July of the same year. A single specimen was collected in the same season at Long Key, at the Dry Tortugas.

Type in American Museum of Natural History.

FAMILY SABELLIDÆ.

Sabella alba n. sp.

Length 45 mm., of which the gills represent 10 mm. Body very slender, not more than 2 mm. in breadth at the collar.

Gills 17 pairs, colorless except for a fine longitudinal dusting of pigment along the bases of some of the rachises; bases of rachises connected by a very delicate membrane. On the outer surface of the rachises beyond the edge of the membrane are minute black ocelli, of varying number on different rachises and on different sides of the same rachis. These seem never to be more than 12 in a row. The extreme tip of each rachis is free from pinnules. The antennæ are slender, sharp-pointed, with (in alcoholic material at least) a noticeable white longitudinal median line. On either side, ventrally, is a patch of fine dark-brown spots similar to those found on the bases of some rachises.

The collar is rather low, its ends widely separated dorsally, but nearly in contact on the ventral surface (plate 3, figs. 10 and 11). The ventral ends have a fine recurved tip, but elsewhere the edge is straight and not recurved. On either side is a ventro-lateral fissure, giving the collar a 4-lobed character.

Body colorless except for a series of dark-brown ventral shields on the somites. Throughout the greater part of its length these shields are divided by a deep, narrow longitudinal fissure. There are 8 thoracic somites.

Thoracic setæ of two sorts, one (plate 3, fig. 12) lanceolate with asymmetrical expansion at the end, the other stouter, enlarged at the apex, shown in side view in plate 3, figure 13, and in full face in figure 14. The setæ of the collar fascicle are lanceolate like those shown in figure 12. Uncinus of thorax with a single tooth and a definite crest of minute denticulations, but without second-

ary teeth (plate 3, fig. 15). The pennoned setæ on the thorax have rather heavy stalks with very delicate, slender terminal portions. The tube is tough chitin, dark brown in color and very difficult to remove from alcoholic specimens without injuring the specimen.

Type in American Museum of Natural History.

Collected in the Dry Tortugas in 1914 and at Guanica Bay, Porto Rico, in 1915.

Parasabella sulfurea n. sp.

Length of body of type 65 mm., 18 mm. of which was in the length of gill.

The gills arise from a rounded basal portion as long as the first 4 body somites. There are about 15 on a side, of nearly uniform length, and with no inrolling on either edge. A very short portion of the apex of each rachis is free from pinnules. For a distance about equal to the length of the basal portion the rachises are united by a delicate web. Beginning just beyond the web and extending to beyond the middle of the rachis each carries on either side of its outer surface a row of minute purple spots. The basal portion of the gills is mostly of a purple color, while their greater part is sulphur-yellow, though an occasional pinnule is purple, but these are too few to affect the general coloration. Tentacles slender, pointed, about twice as long as basal portion of the gills.

A specimen collected in 1909 had, while living, a body bright yellow in color, with a greenish tinge dorsally and some purple markings on the dorsal surface of the collar In alcoholic material the body is colorless, except for the ventral shields, which are a grayish brown throughout.

Collar rather low, its dorsal ends widely separated (plate 3, fig. 16) while ventrally the ends are nearly in contact (plate 3, fig. 17). Each ventral end is prolonged into a triangular recurved lobe, and at the base of the lobe is a pad-like thickening of the collar. The whole collar is very inconspicuous and has a recurved edge.

The torus of the first somite is arranged obliquely just posterior to the dorsal free end of the collar on either side (plate 3, fig. 16). On the next 7 somites the torus is lateral and ventral to the seta tuft. Beginning with the ninth somite and extending throughout the remainder of the body the torus is dorsal to the seta tuft.

Setæ of first setigerous somite of two sorts; one with a long, slender shaft (plate 3, fig. 18), the apex bent and narrowed to an acute point, with an indication of a lateral wing on both the concave and convex sides of the bend: the second form is stouter, with the apex rounded and covered with spines and terminating in a short, slender point (plate 3, fig. 19).

Setæ of later somites of four kinds. In the tuft about equal numbers of slender forms with curved apex, the bent portion covered with spines (plate 3, fig. 20) and of stouter forms with rounded ends (plate 3, fig. 21). In each torus is a single row of uncini, each with a single tooth and a long basal bar. Dorsal to the tooth are numerous fine surface striations, but no indication of secondary teeth (plate 3, fig. 22). In addition, below the uncini is a row of pennoned setæ (plate 3, fig. 23).

Common in the Tortugas, living in a dark-brown chitinous tube, usually in holes in solid coral rock. The tube is usually much longer than the animal and apparently its secretion keeps pace with the deposition of the rock.

Type in American Museum of Natural History.

I have included this in the genus *Parasabella* of Bush (1905, p. 191), distinguished from *Sabella* by the absence of a lateral incision in the collar lobe. In its general appearance and form of setæ it resembles *Protulides elegans* of Webster (1884, p. 325), but differs in the form of the collar.

Metalonome brunnea n. sp.

Length of largest specimen, including gills, 30 mm. Length of gills, 10 mm. Diameter of thorax, 3 mm.

There are about 13 gills on a side, with relatively long pinnules, which have a wavy outline, giving the whole gill a feathery appearance. The antennæ are short, broad at the base, with a very acute tip. The basal membrane of the gills extends for only a short distance up the plumule, and in alcoholic specimens this basal portion is a very dark brown, the color lightening very gradually toward the colorless apex.

Ends of collar widely separated on the dorsal surface, the dorso-lateral portions rather low, the edges not reflexed (plate 3, fig. 24). Ventral to the ventro-lateral notch the collar is produced on either side into a prominent pointed lobe (fig. 25), whose length is more than twice the width of collar. These lobes are in contact along the median line and cover a large part of the bases of the gills. Dorsal to them, between the bases of the gills, is a pair of lip-like folds, whose outer edges are in contact with and possibly are a prolongation of the ventral edges of the collar.

There are 12 thoracic seta-bearing somites. The first seta tuft is near the dorsal end of the collar, and there is no corresponding torus, while on the second and later seta-bearing somites there is a ventral torus. The torus becomes dorsal on the thirteenth setigerous somite. Ventral shields inconspicuous and divided longitudinally throughout the abdominal region by a narrow line.

The setæ of the collar fascicle are long, the terminal portion expanded laterally into a wing which is densely striated on its surface (plate 3, fig. 26, from a profile view). The thoracic setæ are in bundles, those of one side of each bundle being similar in form to the collar setæ, those on the other side having a terminal expansion ovoid when seen in face; between these two is a series graduating in form from one to the other; all have dense striations over the broadened surface. The uncini have a large tooth and a crest of 5 or 6 rows of smaller teeth (plate 3, fig. 27).

The tube is of thin white parchment covered at the end with fine white mud.

Type and cotypes in the American Museum of Natural History.

The specimens were collected at Nassau, British West Indies, by Whitheld.

I was unable to discover any pennoned setæ in the thorax of these forms, and consequently have included them in the genus *Metalonome* as defined by Bush (1905, p. 287).

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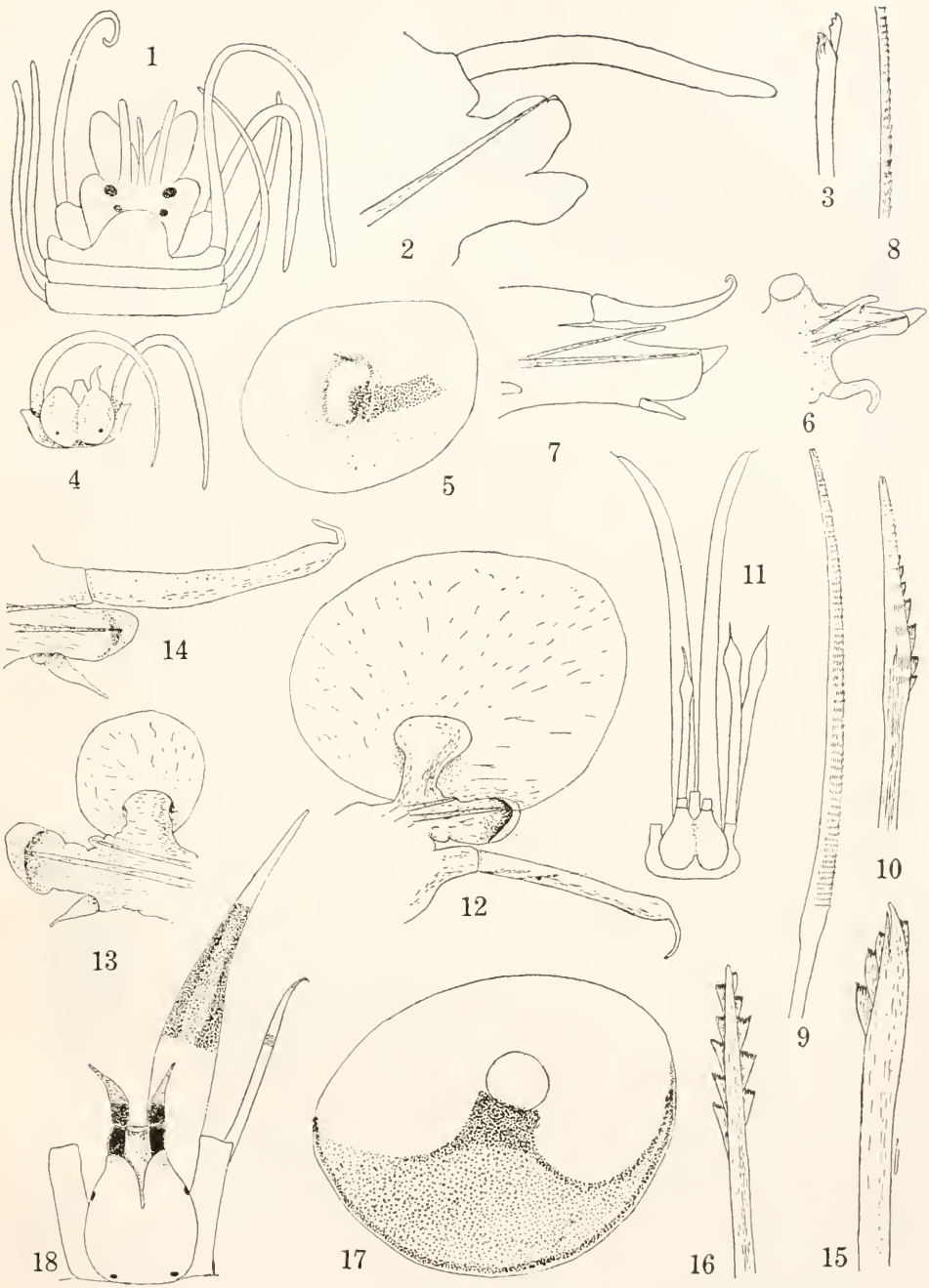


1 to 3. *Odontosyllis octodentata* n. sp.:
1. Anterior end, × 10.
2. Parapodium, ×40.
3. Seta, ×280.

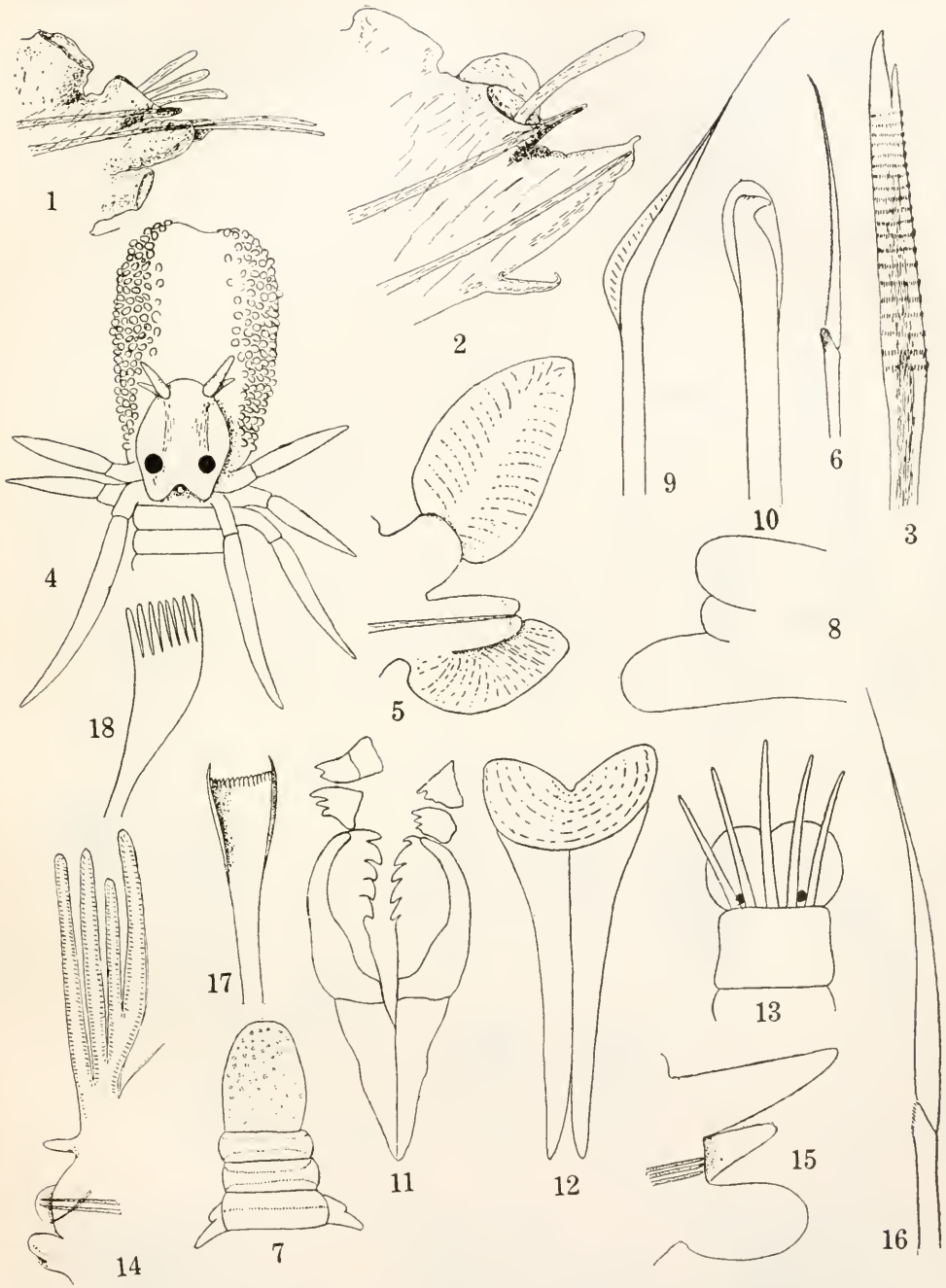
4 to 7. *Lepidonotus inquilinus* n. sp.:
4. Head, ×9.
5. Elytron, ×25.
6. First parapodium, ×28.
7. Middle parapodium, ×28.

8 to 10. *L. inquilinus*—cont.
8. Portion of notopodial seta, ×280.
9. Dorsal seta from neuropodial tuft, ×280.
10. Ventral seta of neuropodial tuft, ×280.

11 to 12. *Lepidasthenia varius* n. sp.:
11. Head, ×6.
12. First parapodium, ×23.

13 to 16. *L. varius*—cont.
13. Eighth parapodium, ×23.
14. Second parapodium, ×23.
15. Lateral view of a seta from an anterior parapodium, ×280.
16. Ventral view of the same seta shown in fig. 15.

17, 18. *Harmothoe variegata* n. sp.:
17. Elytron, ×13.
18. Head, ×8.

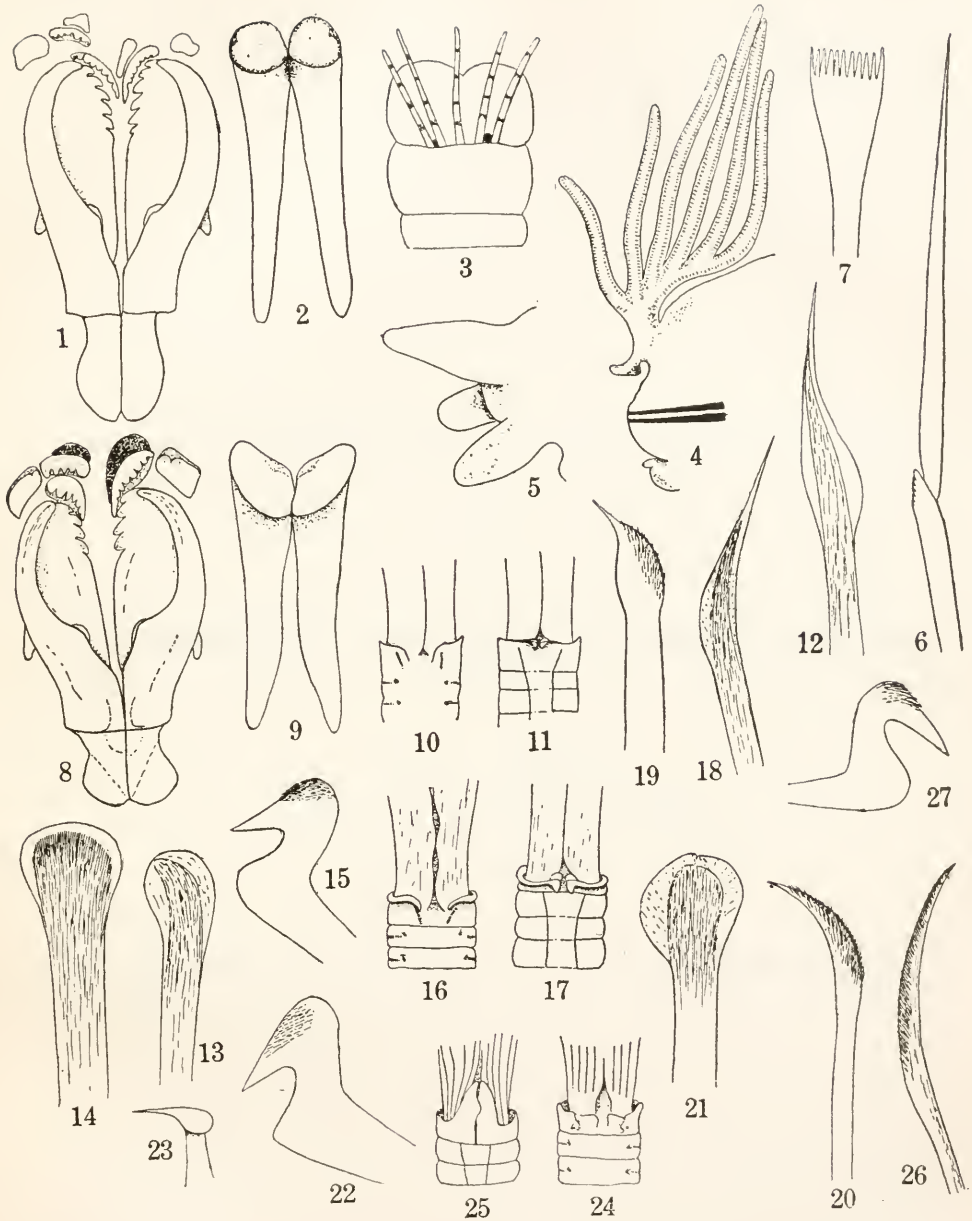


1 to 3. *Harmothoe variegata* n. sp.:
1. First parapodium, ×28.
2. Fifteenth parapodium from the posterior end of body, ×28.
3. Seta from ventral tuft, ×280.

4 to 6. *Phyllodoce tortugæ* n. sp.:
4. Head, ×8.
5. Parapodium from the tenth somite, ×45.
6. Seta from the tenth parapodium, ×280.

7 to 12. *Lumbrinereis cingulata* n. sp.:
7. Head, ×11.
8. Parapodium, ×150.
9. Winged seta from anterior somite showing on lateral view only one wing, ×230.
10. Hooded seta from posterior somite, ×230.
11. Maxillæ, ×58.
12. Mandible, ×58.

13 to 18. *Marphysa viridis* n. sp.:
13. Head and anterior somites, ×7.
14. Parapodium with gills from middle of body, ×38.
15. First parapodium, ×38.
16. Compound seta, ×370.
17. Anterior pectinate seta, ×370.
18. Posterior pectinate seta, ×370.



1 and 2. *Marphysa viridis* n. sp.:
1. Maxillæ, ×19.
2. Mandible, ×19.

3 to 9. *Marphysa nobilis* n. sp.:
3. Head, ×9.
4. 150th parapodium with gills, ×13.
5. First parapodium, ×27.
6. Compound seta, ×232.
7. Pectinate seta from middle of body, ×232.
8. Maxillæ, ×8.
9. Mandible, ×6.

10 and 11. *Sabella alba* n. sp.:
10. Collar and base of gills from ventral surface, ×5.
11. Collar and base of gills from dorsal surface, ×5.

12 to 15 *S. alba*—cont.
12. Lanceolate thoracic seta, ×280.
13. Club-shaped thoracic seta from side view, ×280.
14. Club-shaped thoracic seta from face view, ×280.
15. Thoracic uncinus, ×280.

16 to 19. *Parasabella sulfurea* n. sp.:
16. Base of gills and collar from dorsal surface, ×2.
17. Base of gills and collar from ventral surface, ×2.
18. Longer seta from first somite, ×280.
19. Shorter seta from first somite, ×280.

20 to 23 *P. sulfurea*—cont.
20. Slender seta from second somite, ×280.
21. Rounded seta from second somite, ×280.
22. Uncinus, ×280.
23. Pennoned seta accompanying the uncini, ×280.

24 to 27. *Metalonome brunnea* n. sp.:
24. Collar and base of gill from dorsal surface, ×5.
25. Collar and base of gill from ventral surface, ×5.
26. Seta from collar fascicle, ×280.
27. Thoracic uncinus, ×280.

XI.

THE MICROSCOPIC STRUCTURE OF STRIPED MUSCLE OF LIMULUS.

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Three plates of sixteen figures.

THE MICROSCOPIC STRUCTURE OF STRIPED MUSCLE OF LIMULUS.

By H. E. Jordan.

INTRODUCTION.

The study of the skeletal muscle of *Limulus* was undertaken with two chief objects in view:

(1) To attempt further to test my conclusion suggested by a series of earlier studies on the intercalated disks of vertebrate cardiac muscle, namely, that these disks are properly interpreted as "irreversible contraction bands." If this conception is correct it seems probable that something very similar to intercalated disks may be found in powerfully acting skeletal muscle. Such structures have been repeatedly reported in vertebrate skeletal muscle, but the similarity to intercalated disks is still disputed. It must be emphasized, however, that even certain evidence of the presence of true intercalated disks in skeletal muscle is not crucial for the theory, but only confirmatory. Lack of such evidence can not invalidate the theory as applied to heart-muscle, nor does its possession necessarily prove it correct.

(2) To seek additional evidence in further refutation of the recently revived hypothesis that striped muscle can be interpreted in terms of "muscle-cells" and intercellular myofibrillæ.

A priori, the abdominal muscles which control the caudal spine of *Limulus* seemed to offer very favorable material for the search of the chief data desired. This surmise proved true beyond expectation as respects the second point. Moreover, the material yields very clear-cut data also as to a number of other disputed details concerning muscle structure, chiefly the relation of the ground membrane to the sarcolemma, to the nuclear wall, and to the myofibrillæ.

Concerning the first point no unequivocal evidence accrues. However, this study led to a reinvestigation of *Limulus* cardiac muscle with a different staining technic, and I can now report the presence of a very few, structurally of the simpler type, of intercalated disks. This observation would seem to warrant the generalization that cardiac muscle is characterized, at least as low as *Limulus*, by intercalated disks. The evidence concerning selachii and cyclostomes only remain uncertain. On the other hand, the evidence may perhaps be regarded as confirming the belief of some zoologists that *Limulus* is closely related to the more direct vertebrate ancestors. But similarity of histologic structure need not necessarily have phylogenetic significance; it may

mean nothing more than the result of physiologic similarity. Obviously much comparative microscopic investigation of cardiac muscles is desirable below teleosts.

Other points of considerable interest touch the very close fundamental structural similarity between the spine-muscle and the heart-muscle of *Limulus*, and between the spine-muscle and an early stage of the development of striped muscle in teleosts.

MATERIAL AND METHODS.

As stated above, the material consists of spine and cardiac muscle. The former was collected during the summer of 1914, while engaged in the study of another problem at the Marine Biological Laboratory of the Carnegie Institution of Washington at the Dry Tortugas; the latter was collected at the Cold Spring Harbor Laboratory on Long Island Sound.

The cardiac muscle was fixed in Zimmermann's absolute alcohol-nitric acid solution, and stained with iron-hematoxylin and picric acid-fuchsin. The abdominal (spine) muscle was fixed both in Helly's fluid and in Flemming's strong solution, and also counterstained with the picric acid-fuchsin mixture.

SKELETAL MUSCLE.

GENERAL STRUCTURE.

The mass of muscle in the midline of the abdomen by which the spine (postanal telson) is moved consists of muscle-fibers varying greatly in diameter (fig. 1) and held together by a small amount of delicate wide-meshed connective tissue. The nuclei are scattered apparently promiscuously through the diameter of the fiber; as many as five (fig. 1), or even more, may be seen at approximately the same level in a cross-section of a large fiber. A narrow peripheral sarcoplasmic layer free of myofibrillar material envelops the muscle-fiber; occasionally nuclei occupy this location, even causing the confining sarcolemma to bulge slightly.

Under a magnification of 1,000 diameters, or even less, the fiber in cross-section is seen to have a radially striped appearance shading centrally into a mottled arrangement. Under higher magnification this appearance resolves into broad lamellæ, some apparently extending completely through the entire radius of the fiber. Many of the lamellæ appear split peripherally, producing a deep V- or Y-shaped structure. This represents a radial longitudinal splitting of the lamellar myofibril bundle. Centrally these lamellæ appear to split off smaller lamellæ and cylindric bundles of myofibrils. Between these lamellæ lies the delicate finely granular sarcoplasm (fig. 1B).

Of special interest is the similarity between this adult condition in *Limulus* and an early ontogenetic stage in striped muscle of teleosts.

The structure here described corresponds very closely to that described in the histogenesis of the striped muscle of the trout. It is desirable to know whether these muscle-lamellæ in *Limulus* also trace their origin to a single extranuclear fibril, as is the case in the cyprinoid fishes (Maurer, 1894) and in the trout (Heidenhain, 1913), a matter which is reserved for a future investigation.

Under closer examination these radial lamellæ and central straps and cylinders resolve into fibrils. The unit of structure, then, is the delicate myofibril variously associated into cylinders and lamellæ. These latter are apparently derived from the earlier larger lamellæ by process of splitting in two directions: (1) a radial direction, the splitting being initiated peripherally; (2) a vertical (paratangential) direction, which is chiefly confined to the central area of the fiber.

The limuli from which this tissue was derived were of medium size, *i. e.*, about half-grown. Probably the muscles enlarge by an increase in size of the smaller fibers by the process above described. In very thin longitudinal sections viewed under very high magnification this process of splitting appears to extend to the myofibrils themselves, the assumed units of structure. Indeed, this longitudinal splitting of myofibrils continues to the limit of visibility, and its variously consummated condition gives to the whole a syncytial structure even to its ultimate fibrils (fig. 2B). The impression becomes increasingly strong, as one improves the amplification and the acuity of observation, that the actual ultimate fibrils out of which the visible fibrillæ are formed are ultramicroscopic units—a confirmation thus far of Heidenhain's "Teilkörper theorie" ("histomere" or "protomere" theory), elaborated on the basis of his study of the development of trout skeletal muscle.

Viewed in longitudinal section, different fibers have a very different appearance, depending upon the functional phase of contraction or relaxation. In the uncontracted condition (fig. 2B) the *Q*-disk is conspicuous. Separating two successive *Q*-disks is an area of slightly greater width, very much less deeply stained, the *J*-disk; this is bisected by a deeper-staining but still relatively pale granular membrane, the telophragma (Krause's ground- or *Z*-membrane, 1869). Only the latter spans the intervals between adjacent fibrils; it is therefore a true membrane, as first recognized by MacCallum (1887) for heart-muscle, and as consistently urged by Heidenhain since 1899. The *Q* and *J* disks are regions of differentiation confined to the fibrils. Passing obliquely across the spaces, between adjacent myofibrils, are still more delicate fibrillæ, the ultimate visible units. At the point where the ground membrane and the myofibrils meet the latter appear to swell, giving to the membrane a deep-staining granular appearance; here the ultimate visible fibrils are apparently more closely associated into bundles, the so-called muscle-unit or sarcostyle. There is not the slightest indication in any fiber at any phase of function of any structure suggesting

a mesophragma (membrane of Merkel, 1872; membrane of Heidenhain; *M*-membrane). Such a structure must be lacking in *Limulus* muscle; and in this respect it resembles the wing-muscles of certain insects, and the analogous muscles (pectoral muscles) of birds and bats as described by Thulin.

The fiber in the contracted condition shows only alternating darker and lighter stripes, the latter 2 to 3 times the width of the former. One can not properly speak here of *Q* and *J* disks. The fiber consists of a succession of contraction bands, composed of the ground membrane and *Q*-substance. The dark-staining stripes of contracted muscle correspond topographically to the telophragmata of the non-contracted fiber, but differ from it in that the fibril components are short rods instead of spherical granules.

THE TELOPHRAGMA.

The telophragma, as described above, is a continuous membrane, extending from sarcolemma peripherally to nuclear wall centrally, with both of which structures it is closely united, apparently continuous. Occasionally, where adjacent myofibrils are widely separated, a certain number of the *Z*-membranes can still be seen spanning the gap (fig. 3). The true-membrane condition of the telophragma is further proved by the appearance in certain fibers which have suffered distortion, apparently under pressure applied at right angles to the long axis (fig. 4). Such fibers exhibit a partial folding, the lines of folding (distortion) corresponding always with the telophragmata. The appearance is exactly such as would be expected to result if the fibrils were actually connected with each other by a membrane. The fiber could under the assumed condition, as it does, only fold in the same direction at any level along the lines of the membrane. Such fibers show beautifully also the granular swelling of the fibrils at the level of the membrane.

Centrally the membrane is closely related to the nuclear wall (fig. 3), peripherally to the sarcolemma (fig. 5). To the picric acid-fuchsin mixture the nuclear wall, *Z*-membrane, and sarcolemma have an identical staining reaction—that is, they either do not stain at all or, after long application of the dye, stain uniformly red.

THE SARCOLEMMA.

The muscle-fiber is enveloped by an extremely delicate sarcolemma (figs. 1B, 2A, 4, 5). There can be no doubt regarding its actual presence. Its demonstration is generally difficult in ordinary preparations, due to the very close investure of the endomysium, which is condensed immediately about the fiber. It is only after the study of differentially stained preparations that the distinction between sarcolemma and endomysium becomes marked; after that it can be identified as the innermost layer of the enveloping connective tissue, occasionally lifted away from both the fiber and the endomysium (fig. 4).

Its continuity with the *Z*-membranes marks it as cellular in origin, corresponding to the cell-membrane of the original myoblasts. In picric acid-fuchsin stained material the telophragmata and the sarcolemma remain unstained (appearing dark in such preparations), while the surrounding connective tissue (endomysium) stains a light red. When the staining process is unduly prolonged the sarcolemma also stains red, but then the *Z*-membrane and nuclear membrane and the peripheral sarcoplasm also take on the same red color.

THE NUCLEUS.

The nuclei lie scattered throughout the fiber, at any point on the radii, and are surrounded by a spindle-shaped mass of extremely attenuated sarcoplasm. These sarcoplasmic areas contain a small amount of granular material and several large lipoid spherules. This perinuclear sarcoplasm is bounded by myofibrils which converge apically. There is no suggestion of a cell-membrane. Indeed, that there can be no thought of cell structure is demonstrated by the relation of the telophragmata to the nuclear wall. These two structures are continuous; and the nuclear wall in many instances is drawn out into spines (ridges) at the point of connection with the membrane. In a contracted fiber such projections are less widely spaced, corresponding to the closer apposition of the membranes; in relaxed fibers they are spaced more widely and the projections are less sharp.

CARDIAC MUSCLE.

GENERAL STRUCTURE.

A cross-section of the tubular heart of *Limulus* shows the vast majority of the fibers cut longitudinally. They are collected into larger and smaller bundles, arranged approximately radially, tangentially, and circularly, crossing each other at angles of all degrees; a very few scattered cross-cut fibers may appear peripherally and centrally. The vast majority of the fibers are thus transversely disposed, only an insignificant moiety longitudinally. A median longitudinal section, on the contrary, shows a majority of the fibers cut transversely. Between these, and separating them into wider layers, are narrower bundles of longitudinally cut fibers, the radial fibers. One may search many sections cut near the medial longitudinal plane without seeing a single longitudinal fiber. The latter are certainly extremely rare, but a very few are apparently present, scattered peripherally and next the lumen. Both transverse and longitudinal sections immediately disclose an intricate loose-meshed muscular syncytium.

Figure 6A illustrates a cross-section under low magnification (1,000 diameters) of a large fiber at the level where it has become resolved into several main and a number of secondary branches. Each branch is enveloped by a delicate sarcolemma, the peripheral portion fusing

intimately with the endomysium which incloses the entire bundle and separates the main branches. For purposes of minuter description a smaller trabecula, or branch, may be used. The myofibrillæ are grouped into large masses, in which they are arranged in lamellæ (fig. 6B) undergoing a peripheral radial and a central vertical splitting. The nuclei are located both centrally and peripherally.

Figure 7 illustrates the typical appearance of a small fiber. The myofibrillæ are segregated centrally and are enveloped by a considerable area of granular cytoplasm confined by a delicate sarcolemma. The nucleus here lies wholly in the extrafibrillar sarcoplasm. Upon close inspection the lamellæ are seen to consist of fibrillæ radially arranged. The apparent lamellæ illustrated in figure 6B are therefore smaller groups of fibrillæ.

As in the skeletal muscle, the cardiac fiber consists of peripheral lamellar and central cylindrical groups of fibrillæ. The close similarity between the cardiac and the skeletal muscle extends to the structure and distribution of the nuclei, the finer resolution of the apparent elementary fibrillæ (fig. 8), and the presence of a true cell-membrane or sarcolemma.

In longitudinal sections the appearance of the fiber again, as in the skeletal muscle, and almost identically, varies according to the degree of contraction. Certain fibers (fig. 9) show a wide darker-staining *Q*-disk and an alternating slightly wider *J*-disk bisected by a deep-staining granular *Z*-membrane. In certain other fibers, also uncontracted, only the deep-staining granular *Z*-membrane is conspicuous (fig. 11). The contracted fibers (fig 13c) show only a succession of contraction bands, alternating with lighter-staining disks. The contraction bands are very similar to those of the skeletal muscle and probably likewise represent a thickened *Z*-membrane, due to a segregation of *Q*-substance about them.

THE TELOPHRAGMA.

The telophragma is also a continuous membrane in cardiac muscle. It spans considerable intervals between widely separated adjacent fibrils (sarcostyles) (figs. 10 and 11); at its level the fibrils swell at the point of attachment; it is continuous peripherally with the sarcolemma (figs. 8, 10, 11, and 13) and centrally with the nuclear wall. That the granular appearance is actually due to swelling of the fibrils at the level of attachment is proved by the fact that that portion of the membrane which spans the space between the outermost fibrillæ and the sarcolemma, as also that which spans the perinuclear sarcoplasm, is non-granular (fig. 8). There is no suggestion of a mesophragma.

THE SARCOLEMMA.

That a true sarcolemma actually exists can not be doubted. This conclusion is contrary to that of Meek (1909). He holds that a sarco-

lemma is lacking, its place being taken by a connective-tissue envelope. But conditions are almost exactly like those described for the skeletal muscle. Successful counter staining with picric acid-fuchsin reveals internal to the red-stained endomysium an unstained, or only slightly stained, delicate layer with which the telophragmata are directly continuous. It is very intimately connected with the enveloping connective tissue (fig. 10), but occasionally it may be seen separated therefrom, as in figures 8, 11, and 13. When the staining is prolonged to the point where no distinction can be made on the basis of a difference in staining reaction between the sarcolemma and the endomysium, the telephragmata also, and even the peripheral sarcoplasm, show the same character and depth of stain. If we should decide on the basis of such a picture that the sarcolemma is in fact part of the connective tissue, we would be compelled to draw the same conclusion for the telophragmata and the peripheral sarcoplasm. Moreover, contrary to Meek's observation, the difference, both from the standpoint of size and structure, between the muscle nuclei and the connective-tissue nuclei, is striking, as can be seen from figure 13. The cytoplasm of the blood-cells and of the connective-tissue cells contains lipoid spherules similar to those of the perinuclear sarcoplasm.

THE NUCLEUS.

The nuclei are scattered apparently at random throughout the muscle trabeculæ. To their walls are attached the telophragmata, as in skeletal muscle. Hence, there can be no true cardiac-muscle cells. The cardiac muscle is an intricate syncytium, even to the finest visible fibrillar elements of the myofibrillæ. The most striking difference, and the only other essential difference, barring the gross syncytial character, is the generally much greater length of the nuclei; as many as 8 telophragmata ending on the periphery is a common condition (fig. 12); moreover, it is common to find 4 or more nuclei closely apposed in the same sarcoplasmic area. All the evidence goes to indicate extensive direct nuclear proliferation; all stages in amitosis can be readily demonstrated. Only rarely do 2 closely apposed nuclei in the same sarcoplasmic area appear in the skeletal muscle. This suggests, however, that the method of the nuclear multiplication is the same in the cardiac and skeletal type of muscle in the later growth stages—that is, by amitosis. Not a single karyokinetic figure was seen in the muscle-tissue of either type.

INTERCALATED DISKS.

Meek (1909) reported the absence of intercalated disks in the heart-muscle of *Limulus*. In this conclusion I confirmed him in an earlier study (1912). After reinvestigating the subject with a different staining technic (iron-hematoxylin and picric acid-fuchsin), I am able to report a very few disks of the very simplest or comb type (fig. 15). It

was to be expected that intercalated disks should occur in a heart-muscle essentially so much like that of vertebrate cardiac muscle, with a similar rhythmic beat; and considering the low grade, and the slow rate of heart-beat (32 beats per second, Patten) of the organism, they were expected only in small number and of very simple form. These expectations are realized. However, the extreme rarity of the disks is surprising in view of the fact that they actually occur.

LITERATURE.

The only work known to me on the microscopic structure of *Limulus* muscle is that by Meek (1909) on the cardiac muscle. He describes its structure as a double syncytium composed of trabeculæ "individualized by connective-tissue sheaths"; the peripheral nuclei of the trabeculæ he interprets as *connective-tissue nuclei*. He records also the absence of sarcolemma and of intercalated disks, and notes the close similarity between the heart-musculature of *Limulus* and that of lower vertebrates. Carlson (1904) demonstrated the applicability of the neurogenic theory of heart-beat to *Limulus*. Meek concludes that a syncytial heart-musculature, accordingly, does not necessarily imply the verity of the myogenic theory of conduction. But this functional difference between *Limulus* and vertebrate hearts may in fact inhere fundamentally in the absence of an analogue of the vertebrate atrio-ventricular conducting bundle in *Limulus*.

The gross structural condition of the *Limulus* heart is very significant in this connection. The *Limulus* heart consists essentially of a metameric series of 9 syncytial muscular rings, for the physiologic coordination of which the very scattered peripheral and central longitudinal fibers seem quite inadequate. In the absence of a direct muscular coordinating mechanism the nervous impulse to heart-beat must be conducted by the longitudinal nerve-cord.

Patten (1912) only states that the striped muscle of *Limulus* arises very early from the somites. The histogenetic process is not described, but a detailed description is given of the origin and history of so-called "fiber cells," some of which give rise to definite muscles, others to "semi-amœboid cells resembling blood corpuscles." The original fiber-cells, derivatives of the germ-wall, are said to lie in the first 5 thoracic segments in an intermediate zone median to the germ-wall.

Other problems here considered are touched upon in the following recent works: (1) Baldwin's (1912) on the heart-muscle of the mouse, in which he describes "muscle cells" separated from the myofibrillar substance by a "cell membrane"; this interpretation was shown to be untenable in (2) my paper (1914) dealing with cat and mouse tissue in macerated condition, and by the findings of (3) Asai (1914) in his study of striped-muscle histogenesis in the mouse; (4) Thulin's (1915) work on the wing-muscles of certain insects (Coleoptera), birds, and

bats, from which he records observations which he interprets as indicating the absence of both the meso- and the telo-phragmata, the so-called *Z*-stripe being the only striation discernible and to be interpreted as a contraction band; (5) Heidenhain's work on the histogenesis of striped muscle in the trout, on the basis of which he further supports and extends to striped-muscle tissue his general histologic principle of protomeric analysis, namely, the conception that muscle-tissue is built up by the association into successively larger combinations of ultimate fission elements, the metafibrillæ or protomeres; (6) Jordan and Steele's (1912) comparative study of the intercalated disks in vertebrate heart-muscle, from which it appears that intercalated disks are present in progressively simpler forms in all the vertebrate groups to and including teleost fishes; and (7) my description (1912) of the intercalated disks of hypertrophied human heart-muscle.

DISCUSSION.

The value of the data derived from the study of the *Limulus* muscle depends in degree upon the extent to which they may legitimately serve as a basis for generalization with respect to vertebrate muscle. In the relative simplicity of its striations the *Limulus* muscle seems more like that of vertebrates than like that of arthropods. Moreover, the fundamental close similarity between the cardiac and the skeletal type in *Limulus* is significant, especially as indicating that a main difference between cardiac and skeletal muscle generally is essentially a degree of differentiation, minute morphologic differences following functional differences probably largely inherent in the syncytial arrangement of the fibers in the heart-musculature. In both cases, as in vertebrate heart-muscle generally, the conspicuous stripe is the *Z*-membrane. Accordingly the skeletal muscle of *Limulus* is apparently less highly differentiated than vertebrate skeletal muscle, where the *Q*-disk gives the most conspicuous stripe, a conclusion further supported by the manner in which the nuclei are distributed. In its syncytial character and the presence of intercalated disks the *Limulus* cardiac muscle resembles very closely vertebrate cardiac muscle, a point already emphasized by Carlson and by Meek (8). The infrequency of the intercalated disks precludes an interpretation of these structures in terms of cell boundaries or intercellular substances, or as regions of growth (Heidenhain (12)). The disks consist of rows of modified foci in the fibrillæ. The modification consists of a change characterized tinctorially by an increase in staining intensity for a short distance on one or both sides of the telophragma. Structurally the disk is composed of a series of rod-like areas of the fibrillæ in transverse alinement. The most probable explanation of their formation is that of a change of position of the *Q* (anisotropic) substance from its usual location in relaxed fibers midway between two successive telophragmata, to a

location on either side of these membranes. This explanation answers to the description of the formation of a contraction band. In that a disk appears permanent, it seems appropriately described as an "irreversible contraction band." In view of what was known of the comparative morphology of intercalated disks—coupled with our knowledge of the minute structure of *Limulus* heart-muscle—the simple comb type was to be expected in *Limulus*. This type of disk is actually present. But disks were expected in greater number. Possibly hearts of older limuli would show more abundant disks.

The precise information furnished by the *Limulus* muscle concerning the relation of the ground membrane to the myofibrillæ is of prime importance in respect to the interpretation of occasional serrated disks of apparently normal cardiac muscle and the still more complex disks of hypertrophied heart-muscle. This general type of disk is of very irregular serrated structure. The matter which was difficult of interpretation hitherto was the nature of the cross-connections, in the form of delicate membranes, between adjacent elements of the disk proper. In view of the data now available, namely, the close connection of the myofibrillæ to the telophragmata and the divisibility of the so-called myfibril units into still finer fibrillæ, the matter becomes clear. The essential condition in muscular hypertrophy is an increase in size of the fibers due fundamentally to a longitudinal splitting of the myofibrillæ. Keeping in mind the connection of the myofibrils with the membrane, and the unequal tensions (relative or absolute) under which the adjacent muscle-fibrils work in the hypertrophied heart, the series of changes from the simpler comb type of disks to the complex serrated type of hypertrophied muscle become intelligible (fig. 16).

Heart-muscle, then, is clearly a syncytial structure in vertebrates and *Limulus*, and intercalated disks are presumably invariably present (the matter has not yet received attention below selachii), representing contraction bands which have become incapable of reversion, and thus undergo structural and chemical changes.

We are now in a position also to bring into harmony the discrepant descriptions of the relation of the simpler intercalated disks to the telophragmata. Heidenhain (12) describes the disks as invariably bounded on both sides by a ground membrane. They have also been variously described by other observers as bordered only on one side by a telophragma; and as having no definite relationship to these membranes. My own observations on mammalian cardiac muscle led me to conclude that the disks are generally bisected by a telophragma; occasionally they appear bounded on one side by this membrane; and generally in favorable instances the disks can be seen to shade laterally into a *Z*-membrane. If an intercalated disk of the comb type is correctly interpreted in terms of a contraction band, as I have maintained, then it becomes a simple matter to explain the usual relationship of

the *Z*-membrane to the disk, as also the several occasional variations when the membrane forms either one or both boundaries. The *Z*-membrane forms also the mid-portion of the contraction band. As an "irreversible contraction band," an intercalated disk also is therefore usually bisected by the *Z*-membrane. It may conceivably occasionally happen that only half of the contraction band may be able to relax, whereas the other half may become converted into an intercalated disk. Such a disk would then be bounded only on one side by a ground membrane. If these same conditions should prevail in two successive contraction bands, involving the adjacent halves of the bands, then the resulting disk would be bounded on either side by a *Z*-membrane. Such disks would be rare, the prevailing type being bisected, or bounded on only one side, by the membrane.

The presence of a sarcolemma distinct from the endomysium is proved by the difference in staining reaction of the more delicate inner and coarser outer layers of the closely enveloping connective tissue. The outer reacts to specific stains for connective tissue; the inner does not so react, at least not to the same degree, but always to the same degree to which react also the telophragmata with which this inner layer is in close structural connection. This inner layer is a true sarcolemma. That it represents the original cell-membrane seems proved by the fact that it is connected with the nuclear wall through the telophragmata. The close association of sarcolemma and endomysium must be emphasized; but this is exactly the same as obtains in the case of the skeletal muscle, and exactly the same structural conditions obtain. In neither case can there be any doubt about the presence of a sarcolemma. The fact that one is present in *Limulus* cardiac muscle should go far to remove further skepticism regarding its actual presence in vertebrate heart-muscle.

Neither in the cardiac nor the abdominal-spine muscle of *Limulus* is there any indication of a mesophragma. Thulin records the absence also of a mesophragma, and even of a telophragma, in the wing-muscles of certain insects, birds, and bats. The meaning of this structural peculiarity in these muscles is obscure. Heidenhain (12) claims that a mesophragma is present even in human heart-muscle. The occurrence of such a membrane in cardiac muscle is disputed by many histologists. When apparently lacking, Heidenhain believes that it has a thickness of less than 0.2 micron, the limit of microscopic resolution, hence indiscernible. It seems obvious that the question as to whether heart-muscle anywhere actually contains mesophragmata demands reinvestigation. If it were actually present and of the same nature as the telophragma, and if it had the same relation to the myofibrils and to the sarcolemma, as Heidenhain believes, then it should cause festoons in the sarcolemma like those caused by the telophragma, which is not the case. Furthermore, if present, it should produce a similar folding to

that produced by the telophragmata in certain distorted fibers, as shown in figure 4, which also is not the case.

In this connection should be considered the careful work of Meigs (16) (1908) on the wing-muscle of the fly. He recognizes the existence of at most only 3 different substances in the sarcostyle, that of the *Q* bands and that of the *Z* and *M* lines. He regards *J* as the optical effect of reflection phenomena due to the presence of *Z*. He conceives of *Z* as a substance more highly refractive than its surrounding medium *Q*. His identification of *M* as a true membrane in the wing-muscle of the fly is at variance with the observations on the wing-muscle of beetles by Thulin (4) who records its absence in this group. In *Limulus* striped muscle, both cardiac and skeletal, *M*-membranes can not be discerned either in fresh or fixed and stained material.

In unstained skeletal fibers the *Q*-band is faintly visible; in cardiac muscle it can only occasionally be barely detected. But in stained fibers in certain instances it is distinctly visible in both types of muscle (figs. 6B and 9). Certainly *Q* and *J* have a different staining capacity; and this indicates a structural difference, perhaps inhering only in a relatively greater abundance of "anisotropic granules" in *Q*.

The *J*-band, compared with the *Z*-membrane, is much too wide to permit of any reasonable interpretation of its appearance in terms of refraction phenomena due to the presence of the *Z*-membrane. Moreover, in the contracted fiber one sees only "contraction bands" (apparently $Z + Q$), and *J*-bands, the *J*-band being now topographically in part at the former level of the original *Q*-band. In the case of *Limulus* striped muscle, one is forced to conclude that 3 distinct substances are present, namely, that of the telophragmata and the *J* and *Q* substances.

Limulus increases in size at least throughout early life. Histologically the tissues of the internal organs mature early, but provision must be made for constant enlargement. This fact must be kept in mind in the interpretation of the structure of its muscle. Though mature as concerns its fundamental histogenesis, it presents developmental phenomena. It is of cardinal interest and significance that these are very similar to early histogenetic stages in the muscle of higher forms. In skeletal muscle this point concerns chiefly the arrangement of the fibrillæ in lamellæ and cylinders, each undergoing longitudinal splitting, the former both radial and vertical (paratangential). This is exactly the condition prevalent in developing muscles of the newly-hatched rainbow trout and other teleosts. In cardiac muscle this same point concerns also the nucleus. These nuclei multiply greatly, and by amitotic division. The reason for amitotic multiplication rather than mitotic in the enlarging *Limulus* muscle, both skeletal and cardiac, remains obscure.

This leads naturally again to a consideration of the evidence for "muscle cells" in the sense of Apathy (1888) and Baldwin (1912).

The original myoblasts are lost in a syncytium, in the skeletal muscle appearing as multi-nucleated distinct fibers, in the cardiac muscle as a loose-meshed intricate network of larger and smaller trabeculæ. The muscle-substance here consists of a finely granular sarcoplasm (with large lipoid spherules in the vicinity of the nucleus), throughout which are scattered the myofibrils and the nuclei. The relationship of ground membrane to nuclear wall and to sarcolemma, as also the result of the abundant nuclear amitosis in cardiac muscle, would hardly be intelligible on any other ground. In view of the evidence derived from the study of the muscle from the adult mouse (Jordan), and the data recorded by Asai (3) on the histogenesis of this same heart-muscle, the cellular hypothesis of muscle structure must be definitely abandoned.

If additional evidence were required in refutation of the interpretation of interfibrillar muscle-cells in striped muscle, it may be drawn from the structure of the striped muscles of tunicates (*e. g.*, tail-muscles of *Amaroucium*); here the "fiber" is a mononucleated elongate hexagonal cell, with its nucleus centrally and the myofibrillæ peripherally disposed.

The observations here recorded with respect to the fibrillar structure of *Limulus* muscle, namely, the resolution of the muscular substance into finer and finer complexes to the limit of visibility, strongly support the protomere hypothesis of Heidenhain (5), which maintains that all living matter is divisible into progressively smaller specific units, the ultimate vital molecular units being the "histomeres," "protomeres," or "metafibrillæ."

SUMMARY.

1. Both the skeletal and the cardiac muscles of *Limulus* consist of trabeculæ of finely granular sarcoplasm, holding regularly aggregated collections of myofibrillæ, and confined by a cell-membrane or sarcolemma; throughout the trabeculæ are scattered irregularly the numerous nuclei.

2. In cardiac muscle the main trabeculæ and their branches form a loose-meshed syncytium.

3. Neither type of muscle contains mesophragmata.

4. Very rarely an intercalated disk of the simple-comb type appears in the cardiac muscle.

5. Both types are very similar in respect of the presence and arrangement, in the same phase of contraction, of *Q* and *J* disks, and the telophragmata.

6. The telophragmata are continuous membranes closely attached centrally to the nuclear wall, which is frequently drawn out into projections at the points of attachment, and peripherally to the sarcolemma.

7. The sarcolemma is a very delicate membrane, closely associated with the enveloping endomysium, but reacting differently to specific connective-tissue stains.

8. The evidence is unequivocal against an interpretation of structure in terms of "muscle-cells" and intercellular myofibrillæ.

9. The nuclei of the growing muscles multiply by amitotic division.

10. The myofibrillæ may be resolved into still finer fibrils to the limit of visibility, an observation in support of Heidenhain's "teilkörper" ("protomere") theory.

11. The structurally different constituents of the *Limulus* sarcostyle are the *Z*-membrane and the *Q* and *J* disks. *Q* and *J* most probably differ only in the matter of a relatively greater abundance of certain darker-staining materials, the so-called anisotropic granules, in the former.

12. *M*-membranes occur only in certain more specialized types of striped muscle; they are probably never present in the form of actual membranes in cardiac muscle.

13. In essential structure the cardiac and skeletal muscles of *Limulus* are closely similar, indicating a close functional similarity. The structure serves, moreover, as a splendid illustration of the "law of biogenesis," in that it is practically identical with a stage in the early histogenesis of striped muscle of teleosts.

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DESCRIPTION OF ILLUSTRATIONS.

FIG. 1A.—Transverse section of 5 adjacent skeletal-muscle fibers (abdominal-spine muscles), showing the great variation in diameter and the random distribution of the nuclei (*n*). ×750. Flemming's fixation; iron-hematoxylin stain.

FIG. 1B.—More highly magnified portion (*x*) of the largest fiber shown in A. *n*, nucleus; *s*, sarcolemma; *p*, perimysium (endomysium). The myofibrils are arranged in bundles throughout the sarcoplasm and show a peripheral radial and a central vertical (paratangential) division. ×2,000.

FIG. 2.—Longitudinal sections of: (A) Contracted fiber; (B) uncontracted fiber. *Z*, telophragma; *Q*, anisotropic disk; *J*, isotropic disk. ×2,000.

FIG. 3.—Portions of longitudinal sections of muscle-fibers showing a smaller (*A*) and larger (*B*) nucleus. The perinuclear sarcoplasm contains fine lighter and coarse darker (*g*) spherical lipoid granules. The telophragma (*Z*) is continuous with the nuclear wall, which projects into a series of spines at the points of union. ×2,000.

FIG. 4.—Striped-muscle fiber which has suffered distortion. The lines of distortion are identical with the telophragmata (*Z*), demonstrating the attachment of the myofibrils to the ground membrane. The myofibrils stain more intensely and are slightly swollen, giving a granular appearance at this level, at the point of attachment to the telophragma. *S*, sarcolemma. ×2,000.

FIG. 5.—Portion of longitudinal section of skeletal-muscle fiber showing the telophragmata (*T*) continuous with the sarcolemma. *E*, endomysium; *M*, myofibril; *S*, sarcolemma. ×2,000.

FIG. 6A.—Transverse section of a large trabecula of cardiac muscle, showing the division into several larger and a number of smaller branches. The trabecula and branches are enveloped by a sarcolemma (*S*) in intimate association with the endomysium (*E*). The nuclei are scattered promiscuously throughout the diameter of the fiber. ×1,000.

FIG. 6B.—More highly magnified portion (*x*) of A, showing arrangement of myofibrillæ into lamellar and cylindrical groupings. ×2,000.

FIG. 7.—Transverse section of small cardiac-muscle trabecula, showing the disposition of the larger bundles (*M*) of myofibrils (in which the fibrillæ are grouped in lamellæ and cylinders), the enveloping finely granular, delicate sacroplasm, and the confining delicate sarcolemma (*S*). The nucleus in this section is peripherally placed. ×2,000.

FIG. 8.—Portion of longitudinal section of cardiac-muscle trabecula. The telophragmata are continuous with the sarcolemma (*S*) which is festooned between the membranes. That portion of the membrane to which the myofibrils are attached is granular in appearance; the extra fibrillar portion is non-granular. The sarcoplasm is finely granular. The endomysium (*E*) is very intimately attached to the sarcolemma, but with picric acid-fuchsin counterstain it stains red, while the sarcolemma remains unstained. When the action of the stain is prolonged, endomysium, telophragma, sarcoplasm, and sarcolemma all take on a red or pink color. ×2,000. Zimmermann's fixation; iron-hematoxylin, with picric acid-fuchsin counterstain.

FIG. 9.—Fiber in the uncontracted condition, showing the *Z*, *Q*, and *J* lines. ×2,000.

FIG. 10.—Fiber showing the relationship of the telophragmata and the endomysium (*E*) to the sarcolemma (*S*). The endomysium is red in color, while the sarcolemma and telophragmata remain dark brown or black, in successfully acid-fuchsin stained preparations. ×2,000.

FIG. 11.—Cardiac fiber in the uncontracted condition, showing the deeper-staining granular modification of the myofibrillæ at the levels of attachment to the telophragmata. A peripheral nucleus and the sarcolemma (*S*) are also shown. ×2,000.

FIG. 12.—Heart-muscle nuclei (A and B) showing the relationship of the telophragmata to the nuclear wall. The perinuclear sarcoplasm is bounded by fibrillæ; there is no indication of a cell-membrane. The nuclear wall projects into spines at the points where the ground-membranes are attached. ×2,000.

FIG. 13.—Two adjacent cardiac fibers with intervening connective tissue (*c. t.*, endomysium). *R*, uncontracted fiber; *C*, contracted fiber; *S*, sarcolemma; *b. c.*, blood-cell. Note the difference in size and structure between the nuclei of the connective tissue and the muscle tissue. Both connective-tissue cytoplasm and the perinuclear sarcoplasm contain lipoid spherules. ×2,000.

FIG. 14.—Multinucleated sarcoplasmic area. The division process is amitotic. ×2,000.

FIG. 15.—Intercalated disk of cardiac-muscle fiber of *Limulus*, dividing a contracted (upper) portion from an uncontracted (lower) portion. ×2,000.

FIG. 16.—Diagrams to illustrate the probable derivation of the complex serrated type of intercalated disk characteristic of hypertrophied muscle from the simple-comb type by process of longitudinal splitting of the myofibrils and unequal tensions among adjacent fibrils. The myofibrils are numbered 1 to 5; *Z*, telophragma; *d*, intercalated disk, (A) comb type, (B) type found sparingly in mammalian hearts, and exclusively in hypertrophied hearts. The distortions suffered by the successive telophragmata adjacent to the one directly involved in the disk are not taken into account in the diagram B.

A Hoen & Co. Lith.

LIMULUS SKELETAL (SPINE) MUSCLE

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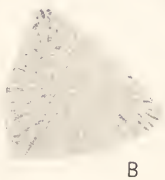

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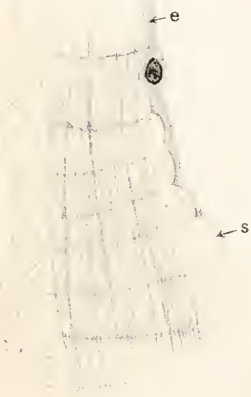

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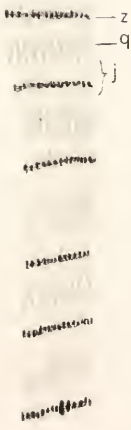

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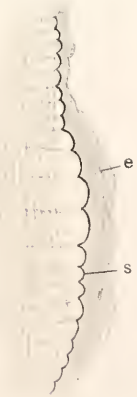

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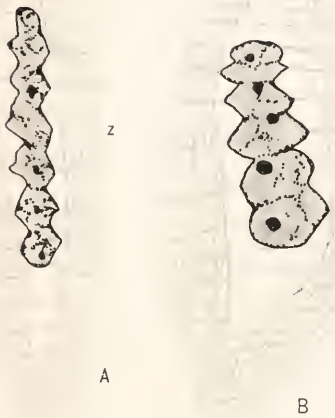
A Hoen & Co Lith.

LIMULUS CARDIAC MUSCLE



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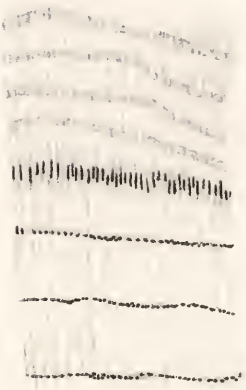

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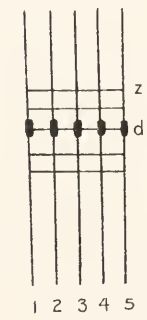

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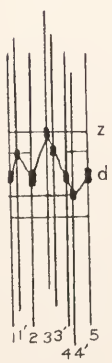

LIMULUS CARDIAC MUSCLE

A.Hoen & Co Lith.

XII.

HEMOPOIESIS IN THE MONGOOSE EMBRYO, WITH SPECIAL REFERENCE TO THE ACTIVITY OF THE ENDOTHELIUM, INCLUDING THAT OF THE YOLK-SAC.

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Five plates of twenty-seven figures.

HEMOPOIESIS IN THE MONGOOSE EMBRYO, WITH SPECIAL REFERENCE TO THE ACTIVITY OF THE ENDOTHELIUM, INCLUDING THAT OF THE YOLK-SAC.

By H. E. Jordan.

INTRODUCTION.

In several recent papers dealing with hemopoietic phenomena in turtle (7) and in pig embryos (8 and 9) I described appearances on the basis of which I maintained a contributive rôle on the part of the endothelium, both intraembryonic and yolk-sac. This position is in agreement with that of Schridde (20), Maximow (11 and 12), and others who have studied the origin and development of blood-cells in various forms. Principally in the work of Maximow and of Dantschakoff (3) this method of partial blood-cell origin has become related to the monophyletic theory of hemogenesis which they support. On the basis of his experimental work with *Fundulus* embryos, narcotized with alcohol, Stockard (21) concludes that the endothelium in these embryos can not transform into hemoblasts; and he views with skepticism the whole mass of morphologic evidence offered in proof of the hemogenic capacity of endothelium, claiming that a different interpretation of descriptions and illustrations is at least as plausible as the one usually given. Stockard, moreover, attacks the monophyletic theory, and in the development of his argument brings the non-hemogenic rôle of endothelium into relation with the polyphyletic theory of blood-cell origin. Moreover, those who believe in the strict specificity of endothelium, and in a degree those who accept the angioblast theory of His, dispute the possibility of endothelium to give origin to blood-cells. It is the chief purpose of this contribution to state and illustrate the evidence which in the opinion of the author justifies a belief in the endothelial origin of some hemoblasts and which agrees to a considerable extent with a monophyletic interpretation of hemopoiesis. This study confines itself largely to the mongoose embryo. No special virtue is claimed for this form in this regard. The chief value of this material lies in the fact of a superb fixation and a favorable staining, and in that it is of a stage of development (5 to 7 mm.) where the phases in question are especially abundant and clear. Moreover, it serves well as a key to the proper interpretation of certain aortic cell-clusters described for the 10-mm. pig embryo. (Emmel (4 and 5); Jordan (9).)

MATERIAL AND METHODS.

The material includes three embryos, with yolk-sac attached, of 5, 6, and 7 mm. length. The embryos were collected in March 1912 at Montego Bay, Jamaica, British West Indies, while with the scientific expedition of the Department of Marine Biology of the Carnegie Institution of Washington, under the leadership of the Director, Dr. Alfred G. Mayer. The embryos were fixed in Helly's fluid, stained *in toto* with Delafield's hematoxylin, lightly counterstained with eosin, and sectioned in paraffin at 10 microns. From the viewpoint of hemopoietic phenomena the three embryos are practically identical. Since the 5 mm. embryo seems to possess a slight advantage in respect of abundance of crucial stages and of differential staining, the following description will pertain almost exclusively to this embryo. The tissue is perfectly normal, as is indicated by the abundant mitoses in practically every tissue. The cell-clusters of the aorta show a progressive increase in size and differentiation from the 5 to the 7 mm. embryo.

HEMOPOIESIS IN YOLK-SAC.

The yolk-sac wall consists of three layers: (1) the thin superficial mesothelial layer; (2) the wide middle mesenchymal layer, filled with endothelium-lined blood-channels; (3) the inner entodermal lining, consisting of a single layer of large cuboidal cells more or less flattened (fig. 2, plate I; and fig. 23, plate III).[1]

The entodermal cells are characterized by the typical cytology described for those of the 10-mm. pig embryo—large vesicular nucleus and a granulo-alveolar cytoplasm frequently containing long, delicate basal filaments. The cytologic evidence indicates a secretory function. A number of the cells are in mitosis.

The mesothelium consists of greatly flattened cells with long oval vesicular nuclei. The mesothelium is in syncytial continuity with the middle mesenchymal layer. Where mesothelium and endothelium abut the two tissues become continuous, and no differential marks, either nuclear or cytoplasmic, appear to identify the two. Cells of either tissue may have larger or smaller nuclei, more chromatic or less chromatic, with a delicately or coarsely granular reticulum, and a more deeply or less deeply staining cytoplasm, depending probably upon the particular phase of modification or function. Exactly the same description will hold also for the mesenchyma. Except for a frequently stellate shape of the mesenchymal cells, the three tissues—mesothelium, endothelium and mesenchyma—are structurally practically identical at this stage. Mesothelium and endothelium are derivatives of the mesenchyma, apparently under the operation principally of the mechanical factor of pressure.

[1]The photomicrographs were made by Mr. William S. Dunn, Cornell University Medical School, New York.

The study of the origin of the blood-cells is best approached by a classification of the different types of cells found free in the blood-vessels, both yolk-sac and intraembryonic. The preponderating type of cell is the erythroblast with pale homogeneous cytoplasm and a spherical vesicular nucleus. The nucleus generally contains one or several nucleoli and a delicate, more or less granular chromatic reticulum (fig. 2). Many of these cells are in mitosis; an occasional cell shows a nuclear condition suggestive of amitosis (fig. 2). The homogeneous cytoplasm has a slightly acidophilic staining reaction; these cells correspond to those of the pig embryo, which, in Giemsa-stained material, have a grayish-pink color. An occasional cell has a slightly larger size than the average of the eythroblasts and is characterized by a finely granular cytoplasm, slightly acidophilic (fig. 12*b*). This is a still younger erythroblast, and corresponds to Maximow's megaloblast type. The granules are more probably the initial hemoglobin content. These cells are transition forms between basophilic hemoblasts and acidophilic erythroblasts. A few cells occur which are characterized by a denser, deeper-staining nucleus and a more highly acidophilic cytoplasm; these represent more differentiated erythroblasts and may be called normoblasts; no non-nucleated erythroplastids are yet present.

Hemoblasts also appear in considerable numbers, more abundantly in the yolk-sac vessels (fig. 1, *a* and *b*). These are characterized by a relatively large granular and vesicular nucleus, usually with several large chromatic nucleoli and a relatively thin shell of basophilic, apparently homogeneous, cytoplasm. In this material not a single mitotically dividing hemoblast was seen. In the pig embryo also a mitotically dividing hemoblast was an extremely rare occurrence. In both instances, however, nuclear amitotic phenomena are abundant. The initial stage is characterized by a kidney-shaped nucleus. The hemoblast may take very irregular shapes (see figure 1*b*) indicating amœboid activity. Figure 1, *c* and *d*, shows two late differentiation stages of a hemoblast; the nucleus stains less intensely, and the cytoplasm has become less basophilic. Figure 1*d* is binucleated, probably the result of a direct division of the nucleus. Trinucleated hemoblasts also are of frequent occurrence.

Giant cells are frequently met with. These differ from the hemoblasts apparently only in their greater size and the irregular character (fig. 1*e*) or the multiple condition of the nucleus. The size relation between nucleus and cytoplasm is slightly altered in favor of the latter. The cytoplasm is basophilic and the nucleus generally stains as intensely as that of the mononucleated hemoblast. The giant cell is most probably a hypertrophied hemoblast. Its multinucleated condition results from amitotic division of the hemoblast nucleus. The multinucleated forms are much less abundant in the 5-mm. mongoose embryo than in the 10-mm. pig embryo. Moreover, in the latter they have

attained a much larger size and may contain many more nuclei than in the former. The evidence from the pig embyro suggested that giant cells were multiple erythroblasts, eventually differentiating into definitive erythrocytes. The giant cells of the mongoose include only the earliest stages of those described for the pig, hence no confirmatory evidence of the above conclusions regarding their erythropoietic function accrues from a study of these cells; but the evidence, so far as it goes, accords with that derived from the study of giant cells of the pig, and the tentative conclusion that they are erythroblasts rather than erythrophages may remain unaltered.

A careful study of the mesenchyma shows that numerous cells, both singly and in groups, take on nuclear and cytoplasmic hemoblast characteristics, and eventually round up more or less and separate from the parent mesenchyme. Exactly similar conditions were described and illustrated for the yolk-sac of the 10-mm. pig embryo and need not be further considered. As single cells these hemoblasts may wander into adjacent vessels; or as groups (blood-islands) they may become inclosed in endothelium forming in the surrounding mesenchyma, to produce an "angiocyst" of the growing vascular net. Similar conditions have frequently been described by various authors (Maximow, Dantschakoff, and others) in sections of the yolk-sac of various forms. Since Stockard has more recently described comparable processes in the yolk-sac of the living *Fundulus* embryo, there need remain no further doubt that mesenchyma does actually differentiate directly into hemoblasts and into enveloping endothelium.

The point of controversy now centers on the question whether the endothelium of the vascular net of the yolk-sac and elsewhere can transform into hemoblasts; for in the *Fundulus* embryo Stockard claims that the endothelium has no hemogenic capacity; and on the basis of this fact he casts doubt upon evidence contributed as a demonstration for blood-cell origin from endothelium in other forms (Schridde, Maximow, Jordan and Flippin, Jordan, Dandy, and others). However, Reagan's (18) more recent findings in *Fundulus* embryos contradict Stockard's conclusions on this point.

Appearances in the yolk-sac of the pig seemed to permit of no escape from the conclusion that endothelium did in fact transform in part into hemoblasts (8). The yolk-sac of the mongoose gives exactly the same evidence. This may be seen by a glance at figure 2*a*, 2*b*, or 2*c*. The cell *b* is at the crucial stage of metamorphosis. It is still continuous with the endothelium, and directly continuous with the endothelial cell *f*; but it has all the nuclear and cytoplasmic characteristics of a hemoblast (compare with 1*a* and 1*b*). Cell *c* represents a further stage in the same process and is about to separate from the endothelium as a mononculeated giant cell. A final stage is represented in figure 3, where a metamorphosed endothelial cell has just become sepa-

rated, and has not yet fully rounded up into the typical hemoblast. Similar examples might be multiplied indefinitely; more are given in the paper dealing with this same phenomenon in the pig embryo; but enough has been shown, especially in the case of cell *b*, to leave no further doubt, I believe, that young endothelium can indeed transform into cellular blood elements.

It is of interest and importance to note that the origin of hemoblasts from endothelial cells also in the bone marrow, both under normal and certain pathological conditions, is described by some of the leading pathologists, *e. g.*, Aschoff (see Mallory's "Principles of Pathologic Histology"). Such a "lining cell" of the marrow blood spaces is believed by Aschoff and by Mallory to be capable of differentiation either into an erythrocyte, a granuloblast, or by hypertrophy into a megakaryocyte, which accords with the evidence derived from my study of the yolk-sac vessels of the pig and the mongoose. Moreover, Mallory, in his list of normal cellular blood elements (p. 21), substitutes for the commonly described mononuclear leucocyte (transitional leucocyte) his "endothelial leucocyte." This cell he derives from "the endothelial cells lining blood, and to a less extent lymph, vessels by proliferation and desquamation. They also multiply by mitosis after emigration from vessels into the lesions."

Since few any longer doubt that young mesenchymal cells can transform on the one hand into certain blood-cells and on the other into endothelium, it seems *a priori* reasonable to suppose that the only slightly altered mesenchyma, the embryonic endothelium, can also occasionally transform directly into hemoblasts. The same thing should be true also, perhaps to a lesser extent, with regard to mesothelium, and for the same reason. According to Bremer (1) the mesothelium covering the body-stalk of a 1 mm. human embryo does in fact give rise to some extent to blood-cells.

The main purpose of this paper is to attempt to establish the thesis that young endothelium has a hemogenic capacity, by showing that intra-embryonically also conditions occur similar to those described for the yolk-sac. These conditions relate to the endothelial origin of hemoblasts, which through a close series of developmental stages can be traced into erythrocytes.

The transition to intra-embryonic conditions next to be described may be made by way of figure 4, which represents a binucleated elongated hemoblast, from a yolk-sac vessel, about to separate from the endothelium with which it is still in part intimately connected and from which it has undoubtedly differentiated. The binucleated cell is apparently also about to divide into two hemoblasts, thus consummating the amitotic process.

CELL-CLUSTERS OF THE AORTA.

The next evidence for hemogenic capacity on the part of the endothelium pertains to certain phenomena in the abdominal portion of the dorsal aorta, the so-called "cell-clusters." Similar clusters in mammalian embryos were first (1909) observed by Maximow (11) in the rabbit embryo.[1] Dantschakoff (3) had already reported comparable structures in the chick embryo. Minot (13) later (1912) described them also for human embryos between 8 and 10 mm. Emmel (4) subsequently (1915) studied more in detail these clusters in pig, rabbit, and rat embryos. At the same time I was making a detailed study of the aortic cell-clusters in the 10 to 12 mm. pig embryos, and had come to essentially the same tentative conclusion regarding their significance as Emmel, namely, that they were masses of hemoblasts differentiating from the endothelium. In the 10-mm. pig embryo these clusters are abundant and very large, some consisting of 100 or more cells; they are practically limited to the ventral portion of the aorta (fig. 24). Proximally they are in continuity with the endothelium and the subjacent mesenchyma. Peripherally they consist of typical hemoblasts and young erythroblasts. The former are characterized chiefly by a deeper-staining basophilic cytoplasm, the latter by a lighter-staining acidophilic cytoplasm. Centrally transition stages occur between endothelium and hemoblast. Occasionally the clusters contain a central core of only slightly differentiated endothelial cells. A number of the cells may be in mitosis, and some exhibit phases in nuclear amitotic division. The mongoose material is of special value in that it shows the earlier stages in the formation of these clusters and thus gives the key to their proper interpretation. The pig material showed a progressive size increase between the 5 and 12 mm. stages of development. The mongoose material shows the same thing. In the 7-mm. embryo the clusters are somewhat larger and the peripheral cells more differentiated than in the 5 mm. embryo.

Figures 5 and 6 show typical small clusters, the first from the ventrolateral wall of the aorta, the latter from the mid-ventral line. Figure 5 gives the appearance of a buckling of the endothelium into the lumen of the aorta, the peripheral cells of the invaginated area assuming hemoblast characteristics. In the cluster, figure 6, the peripheral cells have progressed still further along this line of differentiation, and the proximal pole shows an increase of amitotic proliferative activity and transition phenomena on the part of the endothelium. In the case of the larger clusters the subjacent endothelium has in some instances undergone considerable thickening, being frequently three layers thick.

The close association between the cluster and the endothelium (figs. 25, 26, and 27) through a portion showing a transition between endothe-

[1]Professor Van der Stricht informs me that he had observed aortic cell-clusters in bat embryos as early as 1899; but he claims that the cells of these clusters only differentiate into leucocytes.

lium and hemoblasts, coupled with proliferative activity on the part of the constituent cells, should render unnecessary any discussion as to whether these clusters may not be groups of hemoblasts deposited from the circulating blood and caused by pressure to adhere to the vessel wall. The latter interpretation was given by Minot (13) to these clusters in human and rabbit embryos, in opposition to Maximow (11), who described them in the rabbit as arising by the proliferation of the endothelium. Minot's objection to Maximow's original interpretation was based on his failure to observe either in the human or the rabbit embryos any continuity between the protoplasm of the endothelial cells and that of the "mesamœboids" of the cell-clusters, or any considerable number of mitotic figures in the endothelial cells in the neighborhood of the clusters; "and, finally, because the endothelial nuclei are differentiated, while the nuclei of the cells of the clusters are not differentiated." The mongoose material, however, shows a definite cytoplasmic continuity between endothelial cells and the proximal cells of the clusters. Moreover, while endothelial mitoses are rare in the neighborhood of the clusters, various stages in the amitotic division of endothelial nuclei are abundant. As in the case of certain other tissues of various forms undergoing rapid growth under certain conditions (*e. g.*, blastoderm of pigeon, Patterson; tendon cells of new-born mouse, Nowikoff, etc.), the endothelial cells may here proliferate by the amitotic mode. Finally, the nuclear differentiation of the subjacent endothelial cells is of a lesser degree, judged especially by the small size of the nuclei and their spheroidal shape than that of the more peripheral hemoblast transition elements, many of which contain oval and kidney-shaped nuclei. Further countervailing evidence to the position that the clusters are accretion products from the circulation is detailed in another paper (9) and need not be repeated here.

The aortic endothelium produces not only cell-clusters which contribute hemoblasts and erythroblasts, but single cells also separate from the endothelium in the same manner as described for the yolk-sac. Such cells may appear at any point in the wall of the mesonephric portion of the aorta, though they are more likely to be in the ventral region. A group of two cells appears in the mid-dorsal line of the aorta, subjacent to the notochord. Figure 7 illustrates an endothelial cell along the lateral wall which has rounded up centrally and has assumed hemoblast features; proximally it spreads out in delicate processes for a considerable distance, and is still continuous with the endothelium. Figure 8 represents an endothelial hemoblast separating from the dorsal wall of the aorta. Figure 9 illustrates still another method of separation; this hemoblast is directly continuous on one side with the endothelium. In figure 10 is shown a 2-cell "cluster" of hemoblasts from the lateral wall of the aorta. Figure 11 illustrates

a 3-cell group, located just to the side of the mid-ventral line. This group is still partially attached dorsally to the endothelium and may be regarded as a later step in the process of endothelial hemoblast formation from the yolk-sac shown in figure 4.

In figure 12 is illustrated a 4-cell group from the ventro-lateral wall. Three cells are arranged in horizontal series and jut out directly into the lumen of the aorta; the three cells, moreover, are at successively later stages of differentiation. The proximal cell has intermediate features between an endothelial cell and a hemoblast (*a*); the distal cell has erythroblast characteristics and is very similar to some of the adjacent aortic erythroblasts (*c*). Close to the proximal cell of this group the endothelium is thickened and contains an increased number of nuclei, similar to the attached pole of the multicellular clusters. For purposes of comparison, both dimensional and structural, a few of the adjacent cells of the lumen are shown. A series of successive stages of differentiation is indicated by the letters *a* to *d*; *a* is a typical hemoblast; *b*, a typical megaloblast; *c*, typical normoblasts, and *d*, a late erythroblast (normoblast) stage.

From the foregoing it seems clear that the endothelium of the aorta in the mesonephric portion may proliferate locally in the ventral or latero-ventral or even dorsal wall, giving rise ventrally to cell-clusters which grow in size, the constituent cells of which undergo a coincident differentiation into hemoblasts and young erythroblasts. Similar cell-clusters of larger size are nowhere to be found in the yolk-sac vessels; such are apparently not formed during the yolk-sac homopoiesis; this fact, if countervailing evidence were needed, would sufficiently discredit any interpretation of the aortic cell-clusters in terms of masses of hemoblasts carried to the aorta by the blood and caused to adhere to the ventral wall by reason of pressure and the adhesive properties of their cytoplasm. Coincident with the production of hemoblasts in the aorta through a cell-cluster phase, the endothelium of the same portion of the aorta at any point, though more generally ventrally, may produce also, through the transformation of individual endothelial cells, hemoblasts in an exactly identical manner as above described for the vessels of the yolk-sac. These two processes—hemoblast production by endothelial cell-clusters and by transformation of individual endothelial cells—are essentially similar; the difference involved is one of the degree of endothelial proliferative and differentiative capacity, not one of kind. A few of the cells of the larger cluster appear to have also phagocytic properties.

The question arises as to why the hemogenic activity of the aortic endothelium is generally limited to the ventral area of the mesonephric portion. Why do the larger clusters form only ventrally? This portion differs from other portions in that this is the region along which the larger ventral arterial blood-vessels to the abdominal viscera migrate caudally to their definitive location, the process involving a

shifting of the larger stems (celiac artery, superior and inferior mesenteric arteries) from higher to lower ventral connecting segmental twigs. This region would seem to contain a less highly differentiated type of endothelium—that is, one of greater proliferative capacity, providing thus for an unequal growth between the dorsal and ventral walls. Herein may possibly reside the capacity for some of these endothelial cells to differentiate into hemoblasts. If this interpretation is correct, then the younger endothelium of the intra-embryonic vessels should also be able to transform into hemoblasts. Such youngest type of endothelium should be present in the numerous small blood-channels in the anterior head region next the brain.

ENDOTHELIAL HEMOGENESIS IN INTRAEMBRYONIC MESENCHYMA.

Search for evidence in support of this hypothesis is abundantly rewarded. Figure 13 represents a blood-channel which has just formed out of the pericerebral mesenchyma. The lumen of the channel still contains strands of dissolving mesenchyma. The four cells present represent four different stages in the transformation of a mesenchyme cell into a hemoblast, leading through an endothelial cell. Cell *a* is still typically mesenchymal, but is clearly in process of transformation into an endothelial cell, for two of its processes already form portions of the wall continuous with that portion formed by the typical endothelial cells *b* and *c*. The original endothelial cell now represented by *c* and *d* apparently divided its nucleus, thus forming a binucleated cell; the distal portion of this cell underwent differentiation into a typical hemoblast, while the proximal end represents a transition stage between a typical endothelial cell and a true hemoblast. In this single section of a small area, including four nuclei, the whole process of mesenchymal and endothelial hemogenesis may be seen in abbreviated form; and the process is essentially identical with the manner in which mesenchyma and endothelium function hemogenically in the yolk-sac wall.

Figure 14 is of a cross-section of a capillary vessel including a single endothelial cell, and with a diameter just sufficient to accommodate a single erythrocyte. Since this section is from the same vascularizing area as that of figure 13, the most probable interpretation that suggests itself is in terms of cells *c* and *d* of figure 13. If cell *c*, still continuous with the general mesenchyma, had differentiated into an endothelial cell, and *d* into an erythrocyte, we would have exactly the condition shown in figure 14. The endothelial wall here is still continuous with the mesenchyma, as shown by the strands of protoplasm at the upper pole. If the cytoplasm immediately enveloping the second nucleus of an originally binucleated cell differentiated into hemoglobin-containing protoplasm and thus into an erythrocyte, while the more peripheral cytoplasm remaining in functional association with the second nucleus differentiated into endothelial protoplasm, the

actual condition represented would be realized. A similar complex of erythrocyte and encapsulating endothelium has been seen also in a larger endothelium-lined vascular space.

In figure 15 is shown a hemoblast separating from, but still in cytoplasmic continuity with, the endothelium of a small pericerebral blood-space.

The suggestion has been made that what is interpreted as an endothelial cell rounding up and differentiating into a hemoblast is in reality only an endothelial cell in preparation for mitosis. This is a plausible objection to the interpretation above given and must be met. I have studied many dividing endothelial cells in the pericerebral mesenchyma with this point in mind. It is a fact that both mesenchymal and endothelial cells acquire a deeper-staining cytoplasm just before mitosis; but a mesenchymal cell divides without rounding up and has a relatively smaller nucleus and a less coarse and chromatic nuclear reticulum; moreover, the cytoplasm invariably has a slightly less deep-staining reaction. In these preparations the color of the dividing mesenchymal cell is a deep pink, that of the differentiating hemoblast a brownish or bluish red. The same color difference obtains between the dividing and the hemogenic endothelial cell. Moreover, the intravascularly dividing endothelial cell contracts only relatively slightly, thus becoming a more or less stout spindle-shaped cell (figs. 16 and 18), but does not round up in typical hemoblast fashion. In figure 18 is shown a long dividing endothelial cell at the late anaphase stage; this more probably represents a lateral sprout from the main vessel which is cut in cross-section, but it shows the typical stout-spindle character of the dividing endothelial cell in contrast to the shorter and more spheroidal condition of the differentiating endothelial hemoblast.

The endothelium of these pericerebral vascular channels has considerable proliferative capacity, both internal and external (fig. 17). Extravascularly dividing endothelial cells round up more like hemoblasts and may indeed be progenitors of extravascular hemoblasts; but certain minor morphologic differential characters suggest that we may here be possibly dealing with simply slightly modified proliferating mesenchyma.

It appears, then, that young endothelium, whether in the yolk-sac, ventral area of the mesonephric portion of the aorta, or in the intra-embryonic mesenchyma (pericerebral), functions in the formation of hemoblasts, and in an essentially similar fashion.[1]

[1]The following recent observations have a special bearing upon this point: Huntington (Amer. Jour. Anat., vol. 16, p. 290, 1916) records for certain mammalian embryos that "other red cells develop by the direct transformation of the border endothelial cells lining the early lymphatic spaces." Reagan (Anat. Rec., vol. 10, p. 111, 1915) states that in "chemically treated teleost embryos" (*Fundulus heteroclitus*), "both the endocardium and myocardium have in this region become completely transformed into strongly eosinophilous erythroblasts." Certain investigators have described peculiar mesodermal "fiber cells" in *Limulus* and in spiders and scorpions which on the one hand transform into striped muscle, and on the other into "a special type of blood-corpuscle" or perhaps true blood-cells. (Patten, The Evolution of the Vertebrates and their Kin, p. 235.)

From the above there seemed to be indicated a hemogenic capacity also on the part of the endothelium of the mesonephric glomerulus. This region was carefully studied. But there is apparently no intravascular differentiation of endothelium into hemoblasts. An occasional cell has the appearance of being at an early phase of separation from the vascular endothelium; but in consequence of the irregularity of the plane of section, due to the delicate nature and the contorted condition of the glomerular capillaries, a confident interpretation seems impossible. The normal hemogenic capacity of the glomerular endothelium is certainly very meager, if not actually *nil.*

In an earlier paper (8) I stated the conclusion that in the pig embryo the glomerular endothelium liberated cellular elements (hemoblasts) extravascularly. Study of the sections of the mongoose embryo (of relatively younger stages of development) compels a revision of this conclusion. In the mongoose embryo the visceral layer of the Bowman's capsule of the mesonephric tubule, while closely applied to the glomerulus, is nevertheless easily distinguishable from the endothelium of the glomerular capillaries. The endothelial cells are flat plates; the cells of the capsule are cuboidal or pyriform in shape. An occasional cell appears between the two layers. This may be an endothelial cell separating extravascularly, but such interpretation must remain uncertain.

In the pig embryo the capsular epithelium is still more closely applied to the glomerulus, and the cells of both layers are very similarly flattened. Generally the endothelial nucleus is more vesicular than that of the capsule cell. The cells of the capsule are frequently rounded up and appear to be in early stages of separation. These are the cells which I interpreted as endothelial elements separating extravascularly; but in the light of conditions in the mongoose embryos, this conclusion does not seem warranted. In the mongoose embryo the vascular endothelium of the mesonephric glomerulus is apparently hemogenically inactive; but the presence of the pyriform cells in the capsular membrane (fig. 22), certain of which remain attached by the merest thread of protoplasm—coupled with the fact of an occasional free cell within the lumen of the capsular portion of the mesonephric tubule—suggests that certain of these cells may separate in the embryo to become macrophages. A similar process is described by Mallory in the case of certain infections of the human kidney (*e. g.*, acute capsular glomerulo-nephritis).

It is probably incorrect to regard the endothelium of the mesonephric glomerular capillaries as "embryonic" and relatively undifferentiated. This endothelium has to perform a specialized secretion process in connection with the nephric function of the mesonephros. This may explain the inability normally to produce hemoblasts. On the other hand, the capsular portion of the tubule plays a lesser rôle in this secre-

tory process, and in the mongoose embryo is relatively much less highly differentiated, as indicated by the still cuboidal character of its epithelium. This fact, in view of the mesenchymal origin of this portion of the tubule, may underlie the possibility of a capsular cell to separate from its epithelium and become a free intratubular element, perhaps a macrophage.

CELL-CLUSTERS OF THE PERICARDIUM.

In view of what was said above, regarding the close primary relationship between mesenchyme, endothelium, and mesothelium, it seemed reasonable to expect that mesothelium also might to some extent differentiate hemoblasts, possibly leucoblasts. Careful examination of the pericardium reveals patches of proliferating and differentiating mesothelial cells (fig. 19) very similar to the cell-clusters described for the aorta (compare figs. 6 and 19). These clusters appear both on the visceral and parietal pericardium; and groups of similar cells (syncytia) occur also in the pericardial cavity (fig. 21). The latter most probably are separated portions of the pericardial cell-clusters. Also, single cells may apparently separate (fig. 20) in a manner very similar to that described for the aortic endothelium. All of these cells derived from the pericardium again have many features in common with hemoblasts. It seems quite probable that in the case of the pericardium we are dealing with sources of extravascular hemoblasts (leucoblasts) from the cœlomic epithelium. In the adult it is known that phagocytic leucocytes (macrophages) may arise from the peritoneum, and the same condition may well prevail also in the embryo. In certain experimentally produced teleost hybrids Reagan (18) also describes the transformation of mesothelium into erythrocytes.

DISCUSSION.

The mesenchyma is a fundamental hemogenic tissue. Among its proximate differentiation products are endothelium and mesothelium. These represent originally mechanical rather than functional differentiation products. As such they might be expected to have retained the original differentiative capacity of the parent mesenchyma. The histologic facts above outlined seem to prove that such is actually the case. All the facts detailed are perfectly consistent with this interpretation.

The possibility of origin of hemoblasts from endothelium has become associated with the monophyletic theory of blood-cell origin and contradicts the idea of strict specificity of endothelium demanded by the angioblast theory of intra-embryonic vascularization of His. The opposed ideas of the origin and genetic relationship of the erythrocytes and the leucocytes are expressed in the monophyletic and diphyletic (polyphyletic) theories. It needs to be emphasized that the formulated

processes are still largely in theory. The bulk of the best evidence, however, seems to be in favor of a monophyletic genetic method. The above observations are in accord with, and in a degree a support to, the monophyletic theory. The evidence shows that young endothelium and mesothelium are only slightly altered (mechanically) mesenchyma, and that the two tissues may in early stages give rise to hemoblasts. In the young embryo these hemoblasts differentiate into erythrocytes. The primitive leucocyte ("lymphocyte") is represented by this hemoblast; in later stages this hemoblast (leucoblast) may differentiate into the various types of granulocytes.

The monophyletic theory rests, then, essentially upon these three facts: (1) the origin of the early hemoblast from mesenchyme, now no longer disputed; (2) the differentiation of these hemoblasts into erythrocytes, also no longer disputed; (3) the identity, inferred from a very close structural similarity, of hemoblast and lymphocyte, which latter is regarded as the progenitor of the granulocytes. The third point is the one on which the discussion and dissension center. Granting the verity of the third point, it follows that the primitive leucocytes appear before the erythrocytes. Such an ontogenetic sequence is in accord with the principle of progressive differentiation and with the phylogenetic history—a very significant fact.

The sharpest attack upon the monophyletic theory of recent years has been made by Stockard (21), on the basis of his observations on *Fundulus* embryos; but his more definite and positive facts, namely, the origin of the erythroblasts, "leucoblasts," and endothelium from mesenchyma as seen in the living embryo are actually in accord with the monophyletic theory and furnish strong additional support. The observation upon which he takes foothold to launch his attack, namely, the segregation of erythropoietic and leucopoietic foci, is open to the criticism of misinterpretation with respect to the latter. At any rate, his illustrations of primitive "leucocytes" do not inspire confidence; the karyorrhexis shown would alone make one suspicious of degenerative phenomena, and their general appearance raises the question whether they are not actually (as also Reagan (16) suggests) degenerating erythroblasts suffering nuclear changes and hemolysis.

ADDENDUM.

The above was ready for publication in essentially the form here given early in March 1916. Meanwhile Emmel published a detailed description of the aortic cell-clusters in pig embryos (Amer. Jour. Anat., vol. 19, 3, 1916) and announced the publication of a paper "Concerning certain cellular elements in the cœlomic cavities and mesenchyma of mammalian embryos" (Amer. Jour. Anat., in press for vol. 19). After reading the first of these papers it seemed to me better to note and discuss them in an addendum than to rewrite

my article on mongoose hemogenesis and attempt an incorporation of this discussion in the body of a revised paper. A glance at our illustrations shows the essential identity of our observations; and this point is emphasized by a comparison of our descriptions, after allowance is made for differences in form and degree of development. Our independent observations are in essential agreement, but our interpretations are wide apart. The embryonic derivation of certain cellular blood constituents from proliferating and metamorphosing endothelium seems well established; as is also the origin of leucocyte-like cells (macrophages?) in the serous fluids from the mesothelium of the serous cavities. I incline to view the whole process as a normal hemogenic phenomenon.

Emmel now adheres to a pathologic interpretation, concluding that, while vascular endothelium may not under normal conditions give rise to cellular elements of the blood, "it appears that in both embryo and adult mammals, endothelial tissue ordinarily passive may under certain abnormal conditions, however, assume proliferative activities contributing to the free cellular elements of the circulating blood"; also that "the participation of the mesothelium in the origin of macrophages in the embryonic cœlom is not improbably also a reaction to stimulative conditions arising in part at least through degeneration and disintergration of erythrocytes and other foreign elements escaping into these cavities."

Emmel evidently labors under a feeling of compulsion to interpret his findings in harmony with the original angioblast theory, which denies participation of the vascular endothelium in the normal process of the formation of cellular elements of the blood. It may be quite true that abnormal conditions of various sorts, experimental or pathologic, may stimulate the endothelium to proliferative activity, but recognition of this fact does not compel interpretation of all endothelial proliferative activity in terms of abnormal conditions.

That certain abnormal conditions do stimulate endothelium to proliferation and desquamation and a coincident differentiation proves only that endothelium carries the inherent capacity to thus behave. Similar behavior under early embryonic conditions may be a perfectly normal process. This similarity between a normal embryonic process and an abnormal later condition may be simply an aspect of a very widespread phenomenon in which a pathologic adult condition is an abnormally reawakened normal embryonic condition, *e. g.*, developing cardiac muscle and hypertrophying cardiac muscle, etc. A leiomyoma arises apparently as the result of a normal differentiation of smooth muscle-cells in an abnormal degree. Tumor cells are generally believed to be biologically of the same nature as normal cells. The cell-clusters above described would, if increased and enlarged to an abnormal degree, produce a condition comparable to a hemangio-endothelioblastoma.

Emmel's interpretation of the cause of the formation of free mesothelial derivatives appears especially strained. Why may not the mesothelium normally give rise to the cellular elements of the serous fluids as part of its function, by reason of its close genetic relationship to hemogenic mesenchyma and endothelium? At one point Emmel inclines (p. 402) to identify the cells of the cell-clusters of the aorta with macrophages, and he ascribes the cause of their formation to the presence of possible toxins produced by the "atrophying" and "degenerating" ventral aortic vessels in close association with which some of the cell-clusters are found. This conclusion is based upon a number of fundamental uncertainties. In the mongoose material the cell-clusters consist of typical hemoblasts (mesamœboids); this is in agreement with both Maximow's findings for rabbit and Minot's observations on human and rabbit embryos. The endothelial proliferation products of the embryo need therefore not be confused with macrophages. Moreover, Weidenreich derives the macrophages of Metschnikoff from leucocytes, while Evans avers that macrophages and leucocytes have no direct genetic relationship. On the other hand, many hematologists derive the leucocytes from the common blood mother-cell, the hemoblast, which may have an endothelial origin. A second uncertainty involves the manner of the caudal progression of the celiac, the superior mesenteric, and the inferior mesenteric arteries. Evans inclines to explain the process on the basis of an unequal growth between the dorsal and ventral portion of the abdominal aorta, necessitating thus a less highly differentiated endothelium ventrally. Degeneration of certain ventral vessels probably also occurs; but coincident with this atrophy there may be likewise a new origin of vessels in the formation of progressively lower connections with the main ventral stems; and some of the cell-clusters may be related to the newer vessels, as I believe, rather than to the degenerating vessels, as Emmel believes.

There appears no valid reason why the endothelium of the yolk-sac vessels and that of the embryonic vessels should function differently; why in the case of the yolk-sac vessels the endothelium should be capable of metamorphosing normally into hemoblasts, while in the intra-embryonic vessels with young endothelium the stimulative factor to metamorphosis should have to be a pathologic one. If endothelial hemogenesis in the yolk-sac increased in activity coincident with the degenerative processes of the sac, Emmel's interpretation might be more confidently accepted; but this is precisely where it fails. Endothelial hemogenesis is most active in the yolk-sac of the 10 to 12 mm. embryos when hemopoiesis is at its height in these vessels. Subsequently it decreases, and by the 25-mm. stage no separating endothelial cells can be found. Moreover, in the small vessels of the pericerebral region endothelial cells may occasionally separate to become intravascular elements. In short, wherever only slightly differentiated

endothelium appears, blood elements may originate. Again, if abnormal conditions could explain endothelial proliferation and metamorphosis in embryos, then the experimental *Fundulus* embryos of Stockard, in which cessation of heart-pulsation produced stasis in the blood-vessels, and a consequent degeneration of some of the included cells, should show endothelial proliferative activity; but according to Stockard this endothelium is inactive.

Emmel lays much stress upon the connection between certain ventral aortic clusters and intra-arterial cell-masses. Vessels containing such masses are interpreted as "degenerating"; but the evidence that such are degenerating is unconvincing. In a 10-mm. pig embryo cut in transverse section I found a large cluster attached to one side of the mouth of the superior mesenteric artery. A similar condition prevails in the 5-mm. mongoose embryo. These vessels are not degenerating; if cut obliquely similar clusters might appear to be attached to an intra-arterial cell-mass filling the vessel. Emmel's section No. 6 (p. 419) is apparently obliquely cut. The "intra-arterial cell-mass" may be simply attached to one side of the mouth of the vessel. Such conditions occur in both the mongoose and pig embryo, where no sign of atrophy appears in the vessel itself. On the other hand, ventral branches which later disappear can be seen free of cell-clusters. In short, neither the degeneration nor the occlusion of all aortic branches associated with clusters is definitely proved.

Nevertheless, certain aortic branches undoubtedly do disappear in the caudal shifting of the ventral branches, and this process may be of the nature of a degeneration which may liberate a dilute and slowly acting toxic substance comparable to such substances as stimulate the production of endothelial leucocytes in certain pathologic conditions, *e. g.*, the relatively slightly virulent toxins arising from typhoid and tubercle bacilli. Then this effect should either be felt throughout the whole of the abdominal aorta, or there should be a progressively decreasing effect, as indicated by the abundance of the desquamating endothelial elements from the distal to the proximal (aortic) portion of these ventral rami. But neither of these conditions obtain. In the older embryos the clusters and desquamating cells are practically limited to the ventral wall; in the younger embryos (mongoose) where there are fewer atrophying ventral rami, desquamating cells can be found in the lateral and even the dorsal wall, and the ventral cell-clusters are small. The younger the embryo the less differentiated the endothelium. Moreover, the clusters of the older embryos are near the mouths of the vessels, or farthest removed from the site of presumed intensest degeneration; and numerous vessels contain no clusters at all.

In the 7-mm. mongoose embryo several occluded ventral rami appear, comparable to Emmel's figure 7. The lumen of the vessel is

completely filled with hemoblast-like cells similar to those of the aortic clusters, many of which show degeneration stages, principally a karyorrhexis. But the presence of these cells need not be interpreted as the result of the liberation of a toxin by the atrophying ramus. It seems more reasonable to suppose that the ramus contained a cluster of normal hemoblasts as the result of a normal hemogenic capacity on the part of the endothelium of the vessel, which cluster, in consequence of the atrophy and coincident constriction of the vessel, came to occlude the lumen and ultimately to suffer a resultant degeneration. In other words, the occlusion of the lumen of the vessel and the karyorrhexis of the included hemoblasts are more probably secondary effects of the atrophy of the ramus than that the presence of the cells in the lumen is the result of a toxin formed by the degeneration of the vessels and the included cells and operating as a desquamating stimulant upon the endothelial cells of the vessel.

The most damaging countervailing evidence, however, to the interpretation that the endothelial desquamation products are the result of the action of toxins produced by degenerating blood-vessels and blood-cells, consists in the presence (in the 10-mm. pig embryo) of endothelial cell-clusters of hemoblasts, deep within the superior mesenteric artery (that is, in the middle third of its extent), a level which shows no other signs of atrophy or degeneration and which suffers no subsequent change in a possible farther caudal progression of the vessel to its definitive point of attachment to the aorta. Also in the mongoose embryos and in turtle embryos, the endothelium of the superior mesenteric artery is especially active in liberating intravascular cellular elements.

In a study of the aortic cell-clusters in loggerhead-turtle embryos my attention was arrested by peculiar formations in the inferior vena cava at the level of fusion of the subcardinal veins of a specimen of the twelfth day of incubation. These formations consisted of encapsulated spheroidal masses of hemoblast-like cells, and long strings of hemoblasts attached to the endothelium and extending free into the lumen of the vessel. In the case of the encapsulated groups the capsule consists of an endothelioid membrane of greatly flattened cells. The inclosed cells are very similar to those of the naked cell-clusters of the aorta and the superior mesenteric artery. The structure is comparable to Emmel's figure 5, which represents a similar formation in the aorta of a 12 mm. pig embryo. In one instance the mass was continuous with an underlying loose mesenchyma which appeared to be differentiating into hemoblasts. I incline to interpret this structure in terms of my figure 5. If the invaginated area of endothelium had included a considerable portion of the subjacent vascularizing mesenchyma, then the mesenchyma might have outstripped the endothelium in the process of differentiating into hemoblasts, and so forced, through

pressure, the peripheral endothelial cells to continue their further differentiation into definitive endothelium.

As to the endothelial strands of hemoblast-like cells, I incline to an interpretation in accord with the idea that slightly differentiated endothelium anywhere carries the capacity of producing hemoblasts. In a footnote (p. 407) Emmel describes similar strands in the aorta and the proximal portion of the left umbilical artery in the 12-mm. pig embryo which contained the encapsulated cluster, and in a second 12-mm. embryo, and suggests that they may be associated with the fusion of the two original dorsal aortæ.

If one wishes to adhere to an interpretation of these proliferation products of the aortic endothelium in terms of a toxin, one might locate the source of the toxic substance in the mesonephros, where degenerative processes are initiated in the anterior portion; but such a view is again contradicted by the observation that endothelial desquamation products are practically lacking in the glomerular capillaries, though occasionally present near the aortic mouth of the afferent arteriole. The view that we are dealing with a normal hemogenic process related to a relatively undifferentiated condition of endothelium such as would seem to be requisite in the ventral portion of the abdominal aorta, to permit of the shifting of the celiac, superior mesenteric, and inferior mesenteric arteries by almost any reasonably conceivable process, seems to fit all the facts better than the idea of a toxic influence dependent upon a tissue degeneration in redundant atrophying blood-vessels.

In a second paper ("Concerning certain cellular elements in the cœlomic cavities and mesenchyma of the mammalian embryo," Amer. Jour. Anat., vol. 20, 1, 1916, pp. 73–125), Emmel describes the origin of "macrophages" from the pericardial and peritioneal mesothelium; he describes and illustrates also certain free cell-masses in the pericardial cavity comparable with my illustration (fig. 21) in the mongoose embryo. The causal factor is again assumed to be a toxic substance, in this case liberated in part by degenerating erythrocytes in the cœlom. But it would seem quite as reasonable to regard this formation of macrophages in the embryo as a normal incident in the life of the embryo.

In conclusion, I believe that a careful consideration of all the facts relative to Emmel's observations and my own, upon which there is essential agreement, more amply justifies the conclusion that young, relatively undifferentiated endothelium and mesothelium may anywhere in the embryo normally produce hemoblasts (and macrophages), than that such activity demands the operation of a pathologic factor in the form of a dilute, slow-acting toxin.

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DESCRIPTION OF ILLUSTRATIONS.

All the illustrations, unless otherwise specified, are made from the 5 mm. embryo; the magnification is 1,600 diameters, which is reduced one-third in reproduction.

FIG. 1.—Hemoblasts from yolk-sac vessels: *a*, typical spheroidal type, with basophilic cytoplasm and deep-staining nucleus; *b*, similar cell in amœboid condition; *c*, slightly more differentiated type, with lighter-staining nucleus and cytoplasm (young erythroblast); *d*, binucleated hemoblast differentiated into a young erythroblast; *e*, giant cell (megakaryocyte) with deeply basophilic cytoplasm and irregular, horseshoe-shaped, deeply staining nucleus.

FIG. 2.—Portion of yolk-sac wall, including one large blood-channel fillled with erythrocytes. The mesothelium is shown on the left, the entoderm on the right; *a* to *d* designate successive stages in the metamorphosis of an endothelial cell into an erythrocyte. The cell at *b* has all the nuclear and cytoplasmic marks of a hemoblast, but it is still continuous with the endothelial wall. *c*, a very large hemoblast, also still continuous with the endothelium of the vessel.

FIG. 3.—Hemoblast just after separation from the endothelium.

FIG. 4.—A binucleated elongated hemoblast, still continuous with the endothelium and about to divide amitotically into two hemoblasts. The three large cells at the left are entodermal.

FIG. 5.—Cell-cluster arising in latero-ventral wall of mesonephric portion of aorta through proliferation and differentiation of the endothelium. The peripheral cells have hemoblast characteristics. 6 mm. embryo.

FIG. 6.—A similar small cluster from the ventro-medial portion of the aorta. Proximally the cluster is in syncytial continuity with the endothelium.

FIGS. 7, 8, 9, 10.—Hemoblasts differentiating and separating from the lateral wall of the aorta.

FIG. 11.—A string of three hemoblasts, the cytoplasm of which is still in continuity, and which have differentiated from the endothelium of the latero-ventral wall of the aorta. The proximal hemoblast is still in continuity with the endothelium. 6 mm. embryo.

FIG. 12.—A string of three hemoblasts (*a*) jutting from the endothelial wall into the lumen of the aorta. Within the lumen are shown five erythrocytes; *b*, young erythroblast (megaloblast) with finely granular slightly acidophilic cytoplasm; *c*, normoblasts; *d*, adult erythrocyte. 6 mm. embryo.

FIG. 13.—Binucleated hemoblast differentiating from the wall of a pericerebral blood-channel. The proximal nucleus and its enveloping cytoplasm have endothelial characteristics, and are continuous with the endothelium, which is continuous above with the mesenchyma. The distal nucleus and its enveloping cytoplasm have hemoblast characteristics.

FIG. 14.—Pericerebral capillary with one erythrocyte. This condition may be conceived to have arisen from a binucleated cell as in figure 13, in which one nucleus and the peripheral portion of the cytoplasm differentiated into endothelium, the other into an erythroblast.

FIG. 15.—Hemoblast differentiating from the endothelial wall of a pericerebral vascular space.

FIG. 16.—Pericerebral blood-vessel in which an endothelial cell is dividing internally by mitosis.

FIG. 17.—Pericerebral blood-vessel upon which an endothelial (mesenchymal) cell is dividing externally by mitosis.

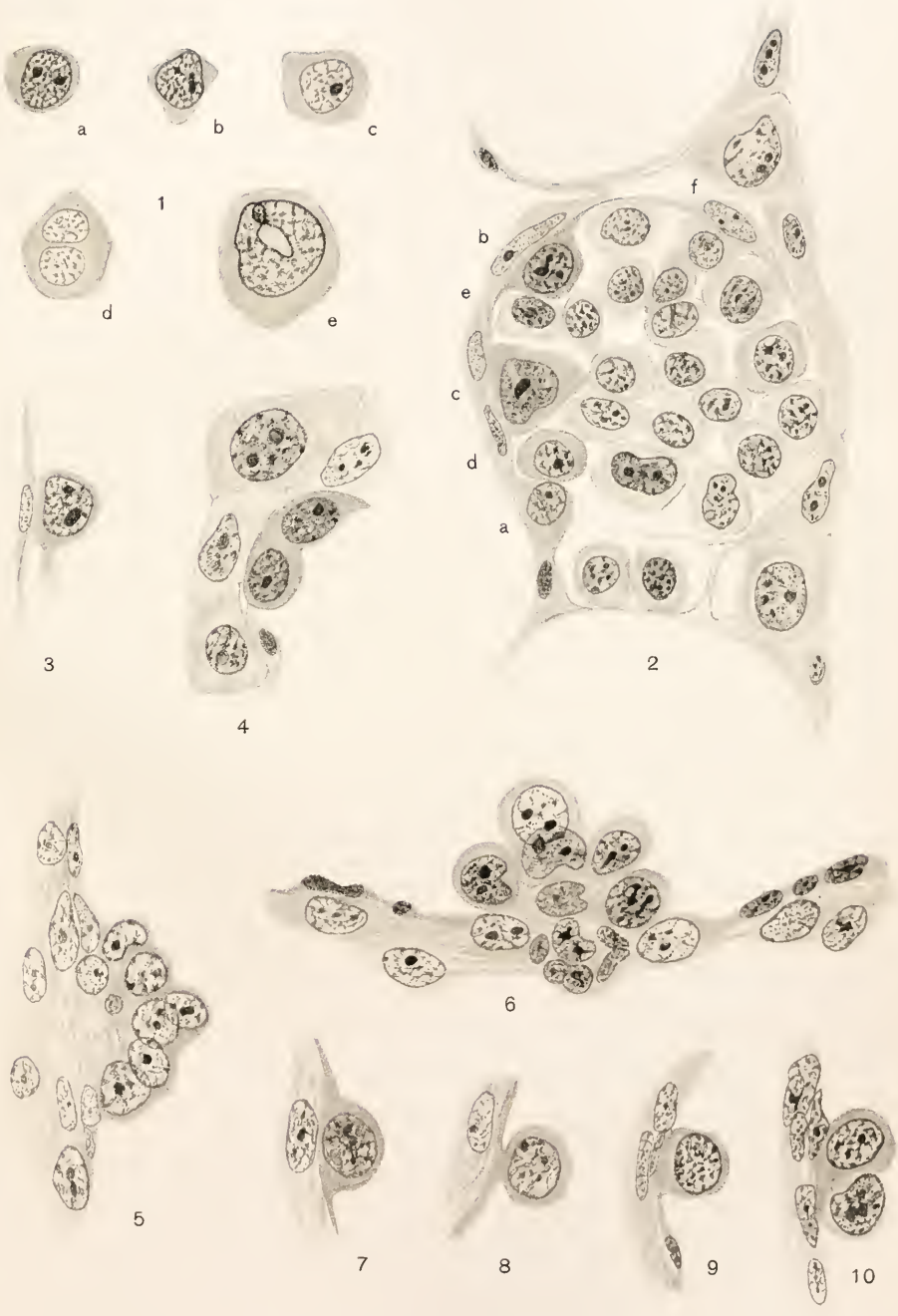
FIG. 18.—Pericerebral blood-vessel in cross-section (containing an erythrocyte) from one point of which is sprouting a lateral vascular twig. The sprouting endothelial cell is at a late anaphase of division. 6 mm. embryo.

FIG. 19.—Cell-cluster of the atrial visceral pericardium. These clusters are similar to those of the aorta, the constituent cells including some with hemoblast characteristics.

FIG. 20.—Hemoblast-like cell separating from the mesothelial layer of the aortic bulb.

FIG. 21.—Free cell-cluster from the pericardial cavity.

FIG. 22.—Portion of visceral layer of capsule of mesonepheric glomerulus, showing cells in process of separation.



HEMOPOIESIS IN MONGOOSE

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HEMOPOIESIS IN MONGOOSE

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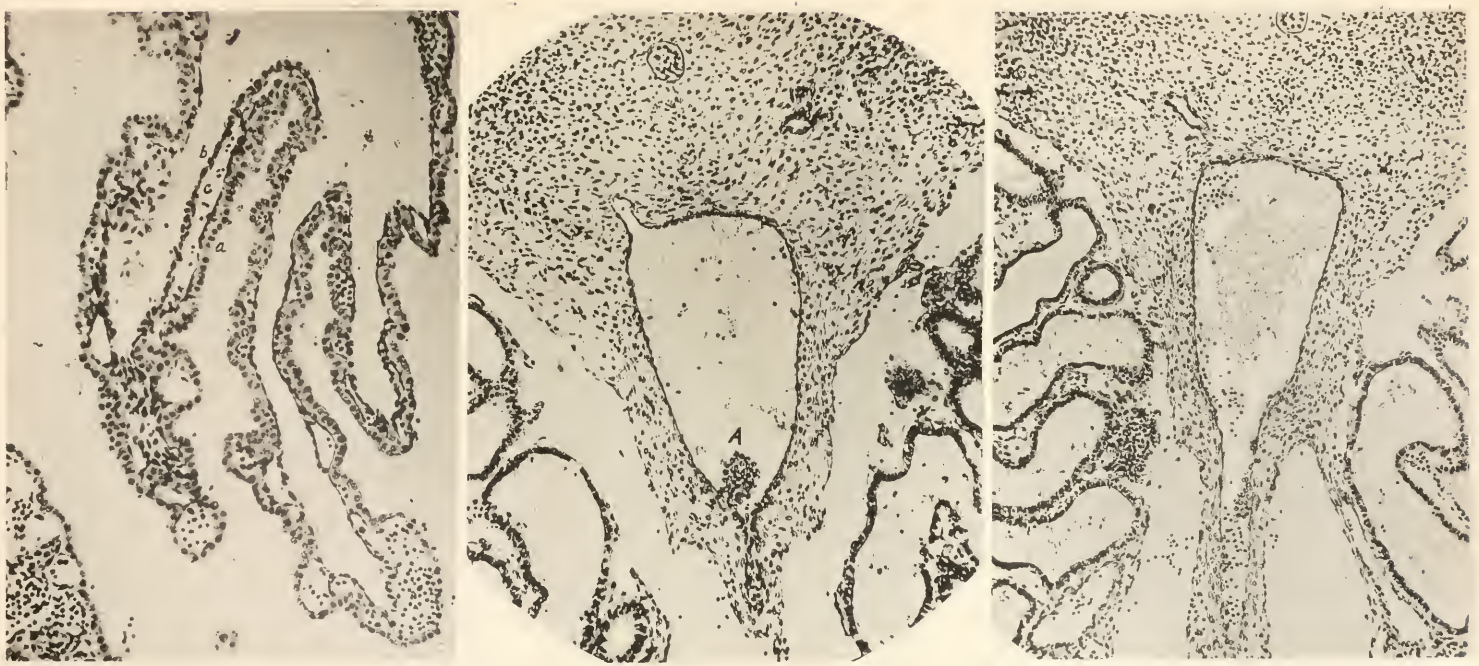
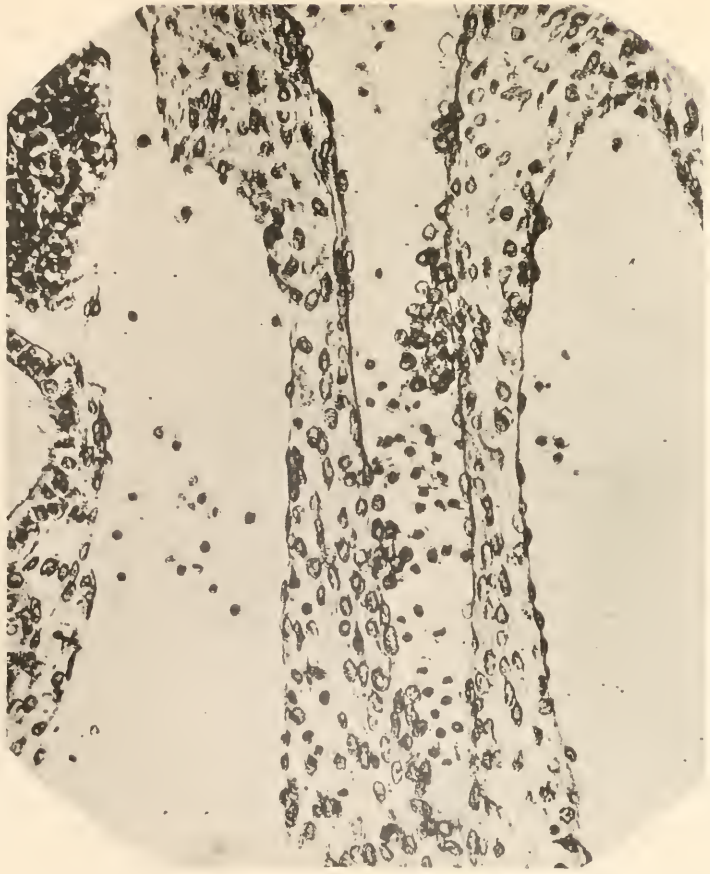


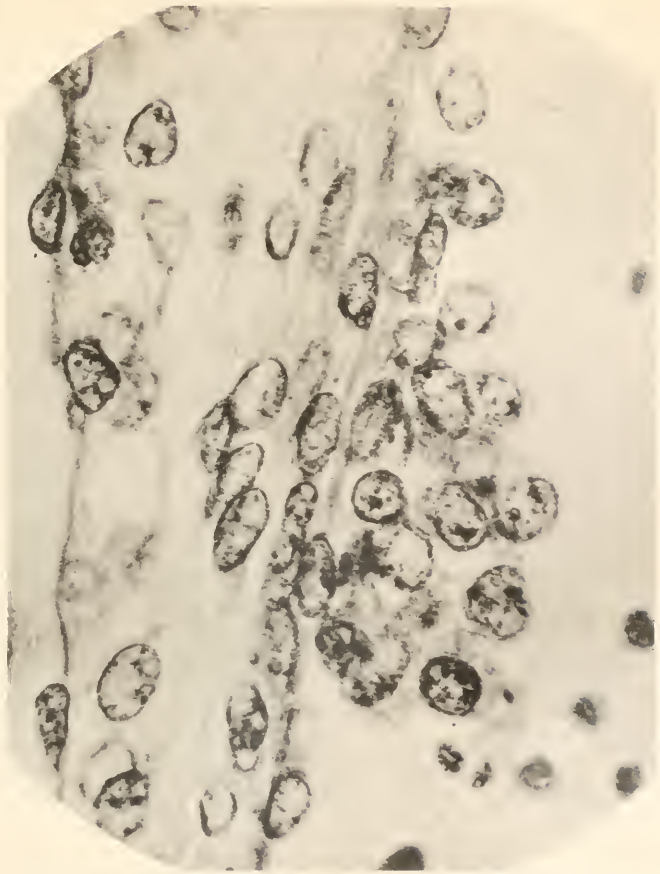
23 Section of portion of yolk-sac wall of 5 mm. mongoose embryo showing, especially well in central upturned loop, character and general relationship of the entodermal lining epithelium (*a*), covering mesothelium (*b*), and blood-space (*c*) filled with erythrocytes. Photomicrograph x 112.

24. Portion of transverse section of a 10 mm. pig embryo at level of first ventral branch below the superior mesenteric artery, showing large cell-cluster (*A*) of hemoblasts and peripheral young erythroblasts within the aorta near the ventromedial line. The proximal cells of the cluster are in cytoplasmic continuity with the endothelium. This cluster extends through 130 microns. Ventral vessel cut tangentially through its caudal wall. The cluster is not in continuity with cells in the ventral branch, nor is there evidence of degeneration in this vessel. Note mesonephros on either side of aorta, and notochord above. Photomicrograph x 85.

25. Portion of transverse section of same embryo (360 microns forward of fig. 24) in the region of the definitive superior mesenteric artery, showing a cell-cluster within this vessel. Three sections caudalward the superior mesenteric artery is open for a length approximately 3 times that here shown. It contains within the middle third other smaller clusters attached to its wall, but there is no evidence of occlusion or degeneration Photomicrograph x 100.

26





27

26. More highly magnified photomicrograph of cell-cluster of fig. 25, showing continuity of proximal cells of the cluster with the endothelium of the vessel, and the hemoblast character of the peripheral cells. Within the lumen of the vessel are shown numerous erythrocytes. x 230.

27. Cell-cluster of superior mesenteric artery shown in figs. 25 and 26. Photo x 500.

XIII.

EMBRYONIC HISTORY OF THE GERM-CELLS OF THE LOGGERHEAD TURTLE (CARETTA CARETTA).

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Six plates of twenty-four figures.

TABLE OF CONTENTS.

	PAGE.
Introduction	315
Material and Methods	316
Descriptive	319
11-day embryo; Flemming fixation, iron-hematoxylin stain	319
10-day embryo; Helly fixation, Giemsa stain	322
12-day embryo; Helly fixation, iron-hematoxylin stain	322
Older embryos; from 13 to 32 days	323
Younger embryos; from 9 to 6 days	325
Youngest embryos; from 2 to 5 days	326
Literature	327
Germ-cell origin in invertebrates	327
Germ-cell origin in vertebrates	328
Discussion and Conclusions	333
Summary of Results	340
Bibliography	342
Description of Illustrations	344

EMBRYONIC HISTORY OF THE GERM-CELLS OF THE LOGGERHEAD TURTLE (CARETTA CARETTA).

By H. E. Jordan.

INTRODUCTION.

The wide discrepancies in the published accounts of the origin and early history of the germ-cells in vertebrates provided the stimulus for the inception and prosecution of this investigation. Thus Waldeyer (1870) described in the 4-day chick embryo the differentiation of germ-cells from the mesothelium covering the mesonephros. This portion of the cœlomic epithelium he designated "germinal epithelium." The theory of germ-cell origin from the mesothelium of the genital ridge early received the indorsement of Semper (1875). Semon (1887) also claimed to have seen a number of cells undergoing metamorphosis from "germinal epithelium" into germ-cells. This theory still forms the basis of the almost universal text-book account, and claims adherents among very recent investigators, *e. g.*, Gatenby (1916).

In 1880, Nussbaum, on the basis of observations on trout and frog embryos, advanced a rival theory which maintained a direct blastomeric origin of the germ-cells and an extra-regional segregation until relatively late stages in the histogenesis of the sexual gland. The central idea of this theory was later (1886) generalized in Weismann's hypothesis of "the continuity of the germ-plasm."

Rückert in 1888 described the origin of the "primordial germ-cells" from a portion of the segmental mesoderm, the so-called "gonotome." Minot (1894) analyzed the available evidence, but adjudged it inadequate to support this novel hypothesis.

In 1892 Hoffman published new evidence, derived from a study of the embryos of various birds, in support of Nussbaum's theory of a germinal path ("Keimbahn") in vertebrates. He developed further the idea that the germ-cells are early segregated as independent elements, *sui generis*, and that these find their way from among the cells of the entoderm to the cœlomic epithelium covering the genital ridge. An increasing number of investigators (*e. g.*, Eigenmann, 1892; Woods, 1902; Allen, 1906, 1907, 1911; Swift, 1914, 1915, 1916) are adding evidence in extension of Hoffman's observations and in accord with the segregation theory of Nussbaum.

In the chick, Swift (1914) traces the origin of the germ-cells back to the entoderm in a peripheral crescentic area in the proamniotic region of the primitive-streak stage, from whence they are described

as migrating by way of developing blood-channels to the splanchnopleure and thence, by amœboid activity, to the primitive gonads.

In the lizard *Lacerta agilis*, von Berenberg-Gossler (1914) follows the origin of the so-called "primordial germ-cells" ("entodermal wandering cells"—Danchakoff, 1908) to the entoderm of the open gut; but he denies for them a direct genetic relationship to the definitive germ-cells, and regards the whole process as a late phase of mesoderm derivation from entoderm contributing largely to the formation of the mesonephric (Wolffian) duct.

Still others (*e. g.*, Felix, 1906, 1911; Dustin, 1910; Firket, 1914) admit the occurrence of primordial germ-cells in the entoderm and their migration in part to the developing genital gland, but attribute to this process only a phylogenetic significance, an ontogenetic reminiscence of an earlier phylogenetic experience; and they claim that these primitive sexual elements early degenerate and are replaced by secondary sex-cells which are differentiated from the mesothelium of the genital ridge.

In view of these discordant conclusions it seemed desirable that the scope of these investigations should be extended to include many more vertebrate forms and additional workers. Among the whole group of investigators Swift was the first to describe, or even to suggest, a vascular route of early migration. Von Berenberg-Gossler (1914) confirms this point for chick and for duck. The possibility seemed to remain, however, that these intravascular so-called genital-cells might indeed be only a type of blood-cell progenitor, perhaps a hypertrophied hemoblast. With this possibility in mind, suggested also by Minot's earlier tentative interpretation (1894) of these cells as elements enlarged in preparation for mitosis, and the later claim of Winiwarter and Sainmont (1909) that they are hypertrophied mesoderm-cells, the work on the loggerhead turtle embryos was planned.

It may be added also that my previous study of the early history of the female germ-cells of the starfish *Asterias forbesii* (1908) had led me to the conclusion that in this form the germ-cells most probably arose by differentiation from the peritoneal epithelium, and so inclined me to a belief in the essential accuracy and general applicability of Waldeyer's original theory. But a steadily augmenting body of cytologic and genetic data would seem to demand a continuous "germ-plasm," which demand does not appear to be met by a derivation of germ-cells from already differentiated soma-cells.

MATERIAL AND METHODS.

The first essential requisite was a complete series of embryos. The second requisite was a favorable staining technic, such as would clearly differentiate the primordial germ-cells at all stages of their early history; which condition was in turn dependent upon the employment

of such a fixative as faithfully preserved differential structural characteristics.

The material embraced in this investigation, selected from a large stock supply, included embryos from the second to the thirty-second day of incubation, distributed, preserved, and stained as follows:

No. of series.		No. of days of incubation.	Fixing fluid employed.	Stains employed.
Cut open behind cephalic tip of pronephros.	2	Germ cells segregated into two bilateral entodermal cords. 2; 5 somites; 2 mm. length.	Helly.....	Iron-hematoxylin and Giemsa.
	2	3; 10 somites; 4 mm. length	...Do.....	Do.
	3	4; 5 mm. length..	...Do.....	Do.
	1	5; 6 mm. length (germ-cells migrating into visceral mesoderm).	...Do.....	Do.
	1	6; 6.5 mm. length (migration to sexual gland begun).	...Do.....	Giemsa.
	1	7; 7 mm. length (open hind-gut beginning to close).	...Do.....	Iron-hematoxylin.
	3	8; 8 mm. length.....	...Do.....	Iron-hematoxylin, and hematoxylin-eosin.
	1	9; 9 mm. length.	...Do.....	Giemsa.
	1	10 (with sharp head and tail bend; 5 mm. greatest length).....	...Do.....	Giemsa.
	1	11.....	Flemming..	Iron-hematoxylin.
	1	12; 9 mm. greatest length.....	Helly.....	Do.
	2	13.....	...Do.....	Do.
	2	16.....	...Do.....	Iron-hematoxylin, Giemsa, and hematoxylin-eosin.
	1	22.....	...Do.....	Delafield's hematoxylin; embryos stained in toto.
	1	25.....	...Do.....	Do.
	1	25.....	Flemming..	Iron-hematoxylin.
	1	32.....	Helly.....	Delafield's hematoxylin.
	25	The sections were cut at from 5 to 10 microns.		

This material was collected on Loggerhead Key, Tortugas, Florida, in the summer of 1914, while at the Laboratory of the Department of Marine Biology of the Carnegie Institution of Washington.

Three nests of eggs were available for the earlier parts of the series. Two additional nests contributed to the later parts of the series. The difficulties of removing the early blastoderm from the yolk were not successfully overcome until the time had passed for preserving stages younger than the end of the second day (5-somite stage). However, this stage shows clearly the origin of the germ-cells from among the yolk-sac entoderm, and the beginning of their segregation into two bilateral cords near the lateral margin of the area pellucida in the caudal half of the blastoderm; and it corresponds closely with von Berenberg-Gossler's illustration (fig. 1) of his youngest lizard embryo (5-somite stage).

Two chief reasons led to the selection of the loggerhead-turtle embryo for this investigation: (1) the possibility of obtaining a large and closely graded series of stages; the number of eggs per nest is near 100; development is comparatively slow, the incubation period being about 8 weeks; (2) I had already familiarized myself in previous works with the embryonic blood-cells of certain *Chelonia* (1913) and also with the male germ-cells in a study of the spermatogenesis of several species of turtles (1914).

The object in using Helly's fluid as a fixative almost exclusively was to preserve the cytoplasmic granules, both albuminoid and lipoid, that is, blood-cell granules and mitochondria, for subsequent differential staining with the Giemsa stain and with iron hematoxylin. Parallel series were prepared with these two stains, with the expectation of facilitating the differentiation between hemoblasts and gametoblasts. This technic was very serviceable, but did not yield quite the fine uniform results hoped for. As concerns the hemoblasts and granular blood-cells (eosinophiles) nothing better could have been desired; but the cytoplasmic contents of the primordial germ-cells were only indifferently preserved. The fixation period should probably have been prolonged for 3 or 4 days beyond the usual 24 hours, especially in the case of the older embryos. Only very rarely were mitochondria and yolk-globules preserved in the later stages. Especially in the germ-cells were the yolk granules dissolved, leaving thus a greatly vacuolated and distorted cytoplasm. But the Helly's fluid and the subsequent treatment involved in the paraffin technic had a very variable effect upon the yolk-granules at different stages. The same type of cell might contain many or only a few, or no yolk-granules. In the latter case the yolk-content had been entirely dissolved. Germ-cells of the 2-day stage of incubation showed many yolk-spherules; by the fourth day no germ-cells contained yolk after this fixation. Obviously the later steps in yolk metabolism were more susceptible after Helly's fixation to the solvent action of the alcohols and oils used in the paraffin technic. The young germ-cells evidently contain less readily soluble yolk than other cells, with the exception of the entodermal cell of the area opaca. In tissues fixed with Helly's fluid and stained with iron-hematoxylin the germ-cells in early stages therefore stand out clearly from among the entoderm-cells of the area pellucida, and accordingly this technic proved to be very favorable for the study of this early, most important, stage.

Some embryos of the eleventh and the twenty-fifth day of incubation were fortunately preserved in Flemming's fluid. It so happens that the 11-day stage of the incubation period is crucial from the viewpoint of the greatest abundance of primordial germ-cells in the area including the closed hind-gut, the mesentery, and the primitive gonad. In these cells mitochondria as well as yolk-granules are well preserved; and so

also are the granules of the confusing large blood granulocytes. The germ-cells in the gonads of the 25-day embryo are practically identical with those of the 11-day embryo in form and size and in regard to cytoplasmic content. Comparative study of the 11-day embryo fixed and stained in the three different ways—(1) Flemming's fluid and iron-hematoxylin stain; (2) Helly and iron-hematoxylin; (3) Helly and Giemsa—revealed admirably the diagnostic marks between the germ-cells and cells with which they might be confused, *i. e.*, blood granulocytes, yolk-laden hemoblasts, and yolk-laden entodermal cells. These marks once established at this stage, earlier stages could be much more readily identified in the Helly-Giemsa or Helly-iron-hematoxylin material than in the Flemming material, since the most confusing and obscuring features were here largely eliminated, namely, the very abundant yolk globules and granules.

DESCRIPTIVE.

11-DAY EMBRYO; FLEMMING FIXATION, IRON-HEMATOXYLIN STAIN.

In view of what was said above it seems clear that the best way of approach to the problem was through the 11-day embryo preserved in Flemming's fluid and stained with iron-hematoxylin. The next step involved a comparative study of embryos of this same stage fixed with Helly's fluid and stained respectively with the iron-hematoxylin and the Giemsa mixtures. Thus the criteria for identifying the primordial germ-cell were established and the previous and later history could then be traced with comparative ease.

This stage corresponds closely with von Berenberg-Gossler's illustration (fig. 9) of a lizard embryo. Primordial germ-cells are present among the entoderm-cells of the closed hind-gut and in the surrounding mesenchyma; in the mesentery, both in the mesenchyma and its mesothelial covering; and in the primitive genital gland, both among the peritoneal cells and in the subjacent mesenchyma (see fig. 2, plate 6). Figure 1, plate 1, is a composite drawing including the several types of cells with which we are now concerned, all from the same immediate region of the mesentery. We may compare first cells *a* and *b*, a typical germ-cell and a typical eosinophil granulocyte, both in the mesenchyma of the mesentery. Cell-type *b* may appear at any stage after the second day of incubation in any part of the yolk-sac or embryonal mesenchyma, including the blood-vessels. At first sight it would seem to correspond to what Swift described as a primordial germ-cell circulating in the blood-vessels of the young chick embryo. It contains an attraction sphere (*i*), a spherical deep-staining chromatic nucleus, and abundant small spherical granules (apparently comparable to the granular mitochondria of germ-cells); but the resemblance is only very superficial and no confusion need arise.

Cell *a* is a typical primordial germ-cell. Ordinarily, in the inactive condition, it has an oval form, rather than the more nearly spherical form of the granulocyte in the same condition. Moreover, it is nearly twice the size of the largest granulocyte. Its nucleus is vesicular, contains a very characteristic finely granular karyoplasm, and stains only very lightly in basic dyes. The radial disposition of the rows of oxyphilic chromatin granules is striking. There is present usually only one large, very chromatic, spherical or oval nucleus; and one (or sometimes several) much smaller chromatic accessory nucleoli; and usually one (sometimes several) pale plastin nucleoli. The cytoplasm is more or less crowded with larger and smaller yolk spherules, staining more or less deeply. It contains also abundant granular, bacillary, beaded, and filiform mitochondria (fig. 2*a*, plate 1).

The granules of the blood granulocyte, on the contrary, are quite uniform in size and in their deep-staining quality. The nucleus of the granulocyte also is relatively smaller, contains a relatively coarse chromatic net-work, and stains much more deeply as a whole in basic dyes. In anticipation of what follows it may here be stated to advantage that primordial germ-cells of earlier stages, including the 2-day stage, are of approximately the size of those of the 11-day stage, only occasionally slightly smaller. The germ-cells are therefore always larger than the largest granulocyte (plate 1, figs. 1*b* and 2*b*), the largest hemoblast (plate 1, fig. 1*h*), and the largest erythroblast (plate 1, fig. 5). The nucleus of the germ-cell also is quite different from that of any of the cells in close proximity to which it may be found—granulocyte (*b*), mesothelium (*M*), mesenchyma, hemoblast (*h*), erythroblast (fig. 5), and erythrocyte (plate 1, fig. 1*e*). It resembles closely only the young entodermal cell, especially in respect of its nucleus, from which cell the germ-cell can, however, be distinguished by criteria which will be given below. Moreover, the granulocyte may assume most varied shapes, at different stages of amœboid progression; the germ-cell, while also capable of amœboid movement, becomes less irregularly modified, extreme forms being represented in figure 3, *aa*, *ab*, *ac*, plate 1.

In the Helly fixed tissue, stained with Giemsa, the granules of the blood granulocytes stain either blue or red, depending upon the phase of development of the cells. The majority of the cells at this stage contain red-staining granules; a few contain only unripe blue-staining granules; and a certain number have mixed granules, including all phases of the ripening process of an eosinophil granule. The nucleus also stains a deep blue. The primordial germ-cell of this tissue, on the contrary, has a light bluish-pink-staining nucleus, with a deep purple nucleolus. The cytoplasm of the germ-cell is vacuolated, the vacuoles being the negative of the dissolved yolk-globules; and it has a coarsely reticular character in consequence, and stains only faintly pink.

Already, from a study of only this stage of development, much information can be gained concerning the fact, route, and method of the migration of the germ-cells from the gut entoderm to the peritoneal epithelium of the gonad. It may be stated here that at no stages studied was any primordial germ-cell, with two exceptions, ever seen in a blood-vessel. The blood-channels in this form evidently do not greatly assist in the transfer of the germ-cells to the sex-gland, contrary to what Swift has demonstrated for the chick; nor has such a route been advocated for any other form except duck (von Berenberg-Gossler); but that the cells do migrate is indicated by the different locations occupied, progressively nearer the genital ridge, corresponding with successively later developmental stages, and that the migration is the result of an inherent amœboid capacity is shown by the various shapes assumed by these cells (figs. 3*aa*, 3*ab*, plate 1); and the path of migration is quite clearly indicated by the pointed condition of the forward end, combined with the altered (flattened and distorted) condition of the cells lying directly in the path of progression. For instance, the primordial germ-cells among the entodermal cells of the closed hind-gut in the 11-day embryo are surrounded by entodermal cells which have become crowded, flattened, and distorted on either side as if through pressure by a cell which enlarged and assumed a spherical shape *in situ*. The similarity in structure and staining reaction between the germ-cells and the entodermal cells, a similarity which becomes progressively closer as earlier stages are approached, seems to speak in favor of an entodermal origin of the germ-cells or a close genetic relationship between entoderm and germ-cells. When the germ-cell lies near the basement membrane of the primitive gut entoderm, the cellular distortion is such as would be caused by a cell migrating from the entoderm into the surrounding mesenchyma. Occasionally a germ-cell can be seen partially within the entoderm and partially within the mesenchyma, an observation which proves a migratory process; and the manner of the distortion of the mesenchymal cells shows that this migration is in a peripheral direction.

Within the mesenchyma of the gut and the mesentery the distortion and flattening of the cells are generally in the direction of the root of the mesentery, thus showing a migratory progression in that direction. Occasional cells, judged by this criterion, are moving towards the mesothelial layer of the mesentery (figs. 3*aa*, 3*ab*, plate 1). Occasional cells are also found among the cells of the peritoneal epithelium of the genital ridge. In the region of the angle between the root of the mesentery and the gonad, and again within the mesenchyma of the sexual gland, the shape of cell and the distortion of the adjacent mesenchymal nuclei are such as to indicate a ventro-medial progression to the surface of the gonad, where the germ-cells come to rest among the peritoneal cells (fig. 2, plate 1). Figure 3*ac*, plate 1, shows a germ-cell similarly located, but almost completely filled with a huge yolk-mass.

The germ-cells are apparently most abundant at about the 11-day stage. This increase is probably merely apparent, due to the segregation of the cells within relatively more restricted and conspicuous limits, viz, gut and mesentery. During earlier stages (to the seventh day), when the gut is still entirely open over the yolk, their distribution is scattered over a wider area; also in later stages, when they have taken their definite position within the enlarged gonad. The number of germ-cells in the 11-day embryo, all situated behind the level of the cephalic tip of the pronephric duct, is approximately 400. Only an occasional cell may be seen at this stage in mitosis. Similarly, during the later stages mitosis is a very rare occurrence. In the stages of 2 to 4 days a few of the germ-cells are in process of division among the entodermal cells. Cells in division are apparently of smaller size and contain relatively little yolk.

10-DAY EMBRYO; HELLY FIXATION, GIEMSA STAIN.

In a 10-day embryo the primordial germ-cells are found in about the same locations; they are slightly smaller, but the smaller size is only apparent, due to a greater cytoplasmic shrinkage in this fluid (fig. 4*a*, plate 1), in part also very probably to the loss of the large yolk-content. Moreover, the Flemming fluid may have caused a slight swelling. The cytoplasm is here vacuolated and coarsely reticular, the result of the solution of yolk-granules. In figure 4, *a*, *b*, *c*, *d*, and *e*, plate 1, are shown primordial germ-cells from the gut, mesentery, and peritoneal epithelium of this stage. For comparison a blood-granulocyte (*f*) is added.

12-DAY EMBRYO; HELLY FIXATION, IRON-HEMATOXYLIN STAIN.

At the twelfth day the germ-cells are still scattered among the entodermal cells of the hind-gut, in the mesentery, and in the genital ridge. Figure 6*a*, plate 1, is taken from among the entodermal cells of the gut; *b*, from among the mesothelial cells of the genital ridge; *c*, from the mesenchyma of the mesentery. The adjacent mesenchymal cells have become changed by the pressure of the migrating cell into flattened encapsulating elements.

The accompanying chart shows the distribution of the germ-cells at this stage, and their condition in different locations. The total number of cells counted is 352, about equally divided between the two gonads (118 in left, 127 in right). A few cells are in mitosis, mostly situated among the entodermal cells. Some are in process of degeneration; such are found chiefly in the mesenchyma of the gut. One cell is situated in what appears to be a vascular space, close to the aorta; another is in process of degeneration within a blood-vessel; seven occur in the periaortic mesenchyma; and four among the neuroblasts of the developing periaortic sympathetic ganglia (fig. 2, plate 4).[1]

[1]The photomicrographs were made by Mr. Wm. S. Dunn, Cornell University Medical School New York.

The periaortic germ-cells migrated dorsally (perhaps only temporarily) instead of laterally across the cœlomic angle, some apparently finding their way into blood-channels. The germ-cells within the gonads are about equally divided between the mesothelium and the subjacent mesenchyma. The mesothelium consists of only a single layer of prismatic cells, greatly flattened where they overlie a germ-cell.

Chart showing the distribution of the germ-cells in a 12-day embryo.

The cells occur from the level of the open gut to that of the end of the closed hind-gut, from slides 11 to 16, a distance of 3 mm. Slides 11 and 12, a distance of 0.8 mm., include the open portion of the hind-gut.

	Gut.		Mesentery.		Genital ridge.		General body mesenchyma.	Total.
	Entoderm.	Mesenchyma.	Mesothelium.	Mesenchyma.	Left gonad.	Right gonad.		
Slide 11..	.....	.....	1 near root.....	1 near root..	....	1	.....	3
Slide 12; open gut figure 1, plate 6.	1 in mitosis; 2 near periphery.	7 dorsally.....	1 at prophase of mitosis; 3 at root; 1 in lower third; 1 near gut end.	1 near root; 2 at gut end.	3	2	.....	24
Slide 13; closed gut, figure 2, plate 6.	2 in mitosis; 5 dorsally.	2 ventrally (degenerating); 3 laterally (degenerating); 3 dorsally; 2 laterally.	.....	2 at gut end; 2 at root end.	9	1	.....	31
Slide 14..	1 in mitosis; 2 degenerating; 7 normal.	5 degenerating; 5 dorsally; 6 laterally; 2 ventrally.	1 near middle point; 1 near root.	1 near root end; 1 near gut end; 1 near middle point.	30	32	.....	95
Slide 15; figure 3, plate 6.	3 near periphery.	1 laterally.....	6 near root end; 2 near gut end; 1 near middle point.	2 near root; 2 near gut.	70	82	.....	169
Slide 16..	1 dorsally...	.....	.....	1 near gut end.	6	9	1 degenerating in blood-vessel; 7 periaortic; 1 in vascular space; 4 among periaortic sympathetic ganglion cells. Figure 2, plate 4.	30
					118	127		352

OLDER EMBRYOS; FROM 13 TO 32 DAYS.

The 13-day embryo is essentially like the one of 12 days (figs. 1, 2, and 3, plate 6), except that relatively fewer germ-cells remain in the gut and more are found within the gonads. In the 16-day embryo (11 mm. in length, from cephalic to caudal bend) the germ-cells have practically all migrated into the gonads, where they come to rest, both among the mesothelial cells and in the subjacent mesenchyma. In the stouter mid-portion of the genital ridge (future gonad), where

the mesenchyma is more extensive, the majority of the primordial germ-cells lie subepithelially; in the cephalic and caudal extremities, on the contrary, where the mesenchyma is sparse, the majority of the germ-cells lie in the peritoneal epithelium, having pressed the columnar cells aside laterally and caused great flattening of the overlying cells. Only an occasional cell is found within the gut and the mesentery. In three slides, including 60 sections, through the hind-gut, three germ-cells were seen in the mesentery (one at the gut end and two in the mid-portion, all apparently in healthy condition), and one in the mesenchyma of the gut. Many of the germ-cells within the gonads at this stage have a coarsely granular nuclear reticulum (fig. 7, plate 2), the granules staining deeply, in contrast to the pale nucleus with very delicate reticulum of the germ-cells of earlier stages.

In figure 7, plate 2, is illustrated a primordial germ-cell from the epithelial layer of the gonad of a 22-day embryo (12 mm. in length). An occasional germ-cell still occurs also in the gut and in the mesentery. The germ-cells of this stage have the same structure and approximately the same size as those from the 10 to 16 day periods, but relatively more cells contain coarsely granular nuclei than in the 16-day stage. The germ-cells of the 32-day stage are no larger than those of the earlier periods, as shown in figure 9, plate 2, of a cell taken from the subepithelial mesenchyma of the gonad. In figure 8, plate 2, is illustrated a germ-cell from the mesenchyma of the mesentery of a 25-day embryo. This particular cell, in common with many others, appears to be encapsulated by flattened mesenchymal-cells. The flattening of the mesenchymal cells is the result of pressure produced by the migrating germ-cell in the direction of the root of the mesentery. It may be emphasized that the germ-cell has undergone no growth or other striking structural alteration between the tenth and thirty-second day of incubation. (Compare figs. 4 and 6, plate 1, with fig. 9, plate 2.) By the sixteenth day all the germ-cells, except a few strays, have left the gut and mesentery and are located within the sexual glands. Also at the 25-day stage an occasional cell may still lag behind in the gut and mesentery; and even in the 32-day embryo several cells were seen in the mesenchyma of the gut, though these were apparently in process of degeneration. The migration process appears to be at its height from the seventh to the sixteenth days; but certain stray cells, mostly degenerating, still persist extra-regionally at the thirty-second day.

In a 25-day embryo, fixed in Flemming's fluid and stained with iron-hematoxylin, where the germ-cells could be more readily detected because their content of yolk-globules was perfectly preserved, 3 germ-cells were seen (in 4 consecutive slides, including 80 sections) in the root of the mesentery—one (degenerating) near the gut end, one in the mid-portion, and one (degenerating) in the mesenchyma of the hind-

gut ventrally. The germ-cells of the embryo, in contrast to all other cells (except liver-cells), still showed abundant yolk-granules and some mitochondria.

The migration period is obviously not sharply limited, but extends in some degree through half the incubation period. Some germ-cells apparently lag hopelessly behind and degenerate, especially in the gut. The initial conspicuous marks of degeneration relate to stages in the disappearance of the nucleus either by solution or karyorrhexis.

YOUNGER EMBRYOS; FROM 9 TO 6 DAYS.

The 9-day embryo (figs. 1 and 2, plate 5) differs from the 10-day embryo only in the relative abundance of the germ-cells at different levels of the migration route; the germ-cells are relatlvely less abundant in the higher levels and in the gonads.

In figure 10*a*, plate 2, is illustrated a germ-cell of an 8-day embryo taken from the splanchnic mesoderm at about the mid-region of the area pellucida. It appears to lie in a lacuna, the product of shrinkage; *b* is taken from the mesentery and is closely enveloped by mesenchymal cells. The latter germ-cell has a diameter of only about 13 microns; *a*, about 16 microns. For comparison, two hemoblasts are included, *c* and *d*. The germ-cells at this stage are most abundant in the mesentery and in the median portion of the splanchnopleure.

In the 7-day embryo the hind-gut is open throughout—that is, beyond the anterior tip of the pronephros. The mesentery is relatively very long caudally. Some germ-cells are found among the entodermal cells medially, but the majority are in the long mesentery, widely scattered, both among the mesenchymal and the mesothelial cells, and in the medial extremity of the visceral plate of mesoderm. A few cells are already found within the genital ridge and a few remain scattered among the entodermal cells of the open gut. In figure 12, plate 2, is illustrated a large germ-cell from among the entodermal cells of the hind-gut, at the point of initial closure. The cytoplasm contains enormous peripheral vacuoles and is much shrunken, due to the dissolution of the yolk-masses of the cell. Figure 11, plate 2, shows a younger germ-cell among the entoderm-cells of the area pellucida, near the mid-line. The difference in size of these two cells (16 microns and 20 microns) might be interpreted to indicate a considerable growth of the germ-cells at about the 7-day stage, but it more probably indicates merely a normal size variation, since large germ-cells appear already at the 3-day stage (10 somites) (figs. 13, 14, and 15, plate 2). Swift (1914) records a variation in diameter of the primordial germ-cells in the chick embryo of from 14 to 22 microns, the average being about 16 microns. In this embryo a few germ-cells among the entodermal cells of the open gut and in the mesothelium of the mesentery are in mitosis.

The body of the 6-day embryo lies almost flat upon the yolk; the gut is open behind the cephalic extremity of the pronephros. Only at this point is there a mesentery, and it is very short and contains no germ-cells. The germ-cells occur in both the entoderm and mesoderm of the open gut, the majority lying scattered in the mesoderm medially.

YOUNGEST EMBRYOS; FROM 2 TO 5 DAYS.

Towards the end of the second day of incubation, when 5 somites are formed, the primordial germ-cells are just becoming segregated within the entoderm of the area pelllucida. The cells now occur in two bilateral cords, more or less oval in transection. These cords extend from just in front of the primitive pit (neurenteric canal) to the caudal end of the primitive streak; they lie laterally in the area pellucida. The germ-cells are not yet as closely aggregated as during the following day. Cells can still be found in process of migration from the yolk-sac entoderm of the area opaca to the entoderm of the area pellucida.

At the 3-day stage (10 somites) the cords of primordial germ-cells are more closely segregated and have apparently moved slightly more medially (fig. 1, plate 3). They now extend from the sixth somite to the caudal extremity of the primitive streak. These cords do not have a uniform diameter throughout, but are more or less interrupted, suggesting a segmental condition, recalling the "gonotome theory" of Rückert.

Figure 15, plate 2, shows a germ-cell from among the yolk-laden entodermal cells of the area opaca of the 3-day stage; an entoderm-cell nucleus is added at the right (*a*) to show the similarity of nuclear condition between the germ and entodermal cells. Figure 14, plate 2, shows a germ-cell taken from the area pellucida. This cell has a diameter of 16.5 microns. Figure 13, plate 2, shows a similar cell in process of migration from among the entodermal cells of the area pellucida into the overlying visceral layer of the lateral mesoderm. Closely similar conditions appear also at the 2-day stage.

The 5-day stage (fig. 2, plate 3, and fig. 1, plate 4) represents a crucial epoch in the early history of the primordial germ-cells. The gut is open throughout and the embryo lies flat upon the yolk. The entodermal cords of germ-cells have made an intimate linear contact with the visceral plate of the lateral mesoderm (fig. 1, plate 4), and are beginning to pass into the latter and to migrate medially. The 4-day stage shows transition conditions between those described for the 3 and 5 day stages. Meanwhile the angioblast layer of the visceral mesoderm is becoming vascularized, and an occasional cell may become inclosed by, or migrate into, a blood-channel, from which it again migrates into the surrounding mesenchyma or possibly later degenerates somewhere in the blood-vessels.

Between the first and sixth days, then, the scattered primordial germ-cells have become segregated from out of the area opaca into a pair of bilateral cords lying near the lateral border of the area pellucida in approximately the caudal half of the embryonic area (fig. 1, plate 3), whence they pass at about the fourth to fifth days into the visceral plate of the lateral mesoderm (fig 1, plate 4), and thence medially towards and into the forming mesentery of the just-closing hind-gut (fig. 1, plate 5). Certain stray germ-cells may persist in any portion of the hind-gut and its mesentery until about the 32-day stage, at which time the final remnant largely degenerates, a few possibly persisting and perhaps furnishing foci for future neoplastic growths.

The very close resemblance between the nuclei of the entodermal cells of the area opaca and the included germ-cells seems to indicate a very close genetic relationship; and their cytomorphic features disclose an equally undifferentiated condition. The only striking difference between these cells is one of shape; the germ-cell is oval, the entoderm-cell very irregular and apparently in syncytial relationship with other entodermal cells.

LITERATURE.

GERM-CELL ORIGIN IN INVERTEBRATES.

The clearest and most striking case of early germ-cell differentiation among the invertebrates is that of *Ascaris megalocephala*, in which Boveri (1892) traced the germ-cells through the cleavage back to the 2-cell stage. The conspicuous differential feature of this cell at the 2-cell stage (and to the sixth cleavage) is the maintenance at mitosis on the part of one of its daughter-cells of the integrity of the chromosomes in contrast with the soma-cell, both of whose daughter-cells suffer a diminution of the chromatin at each division. The germ-cells become segregated at the 32-cell stage of cleavage, after which they produce only similar germ-cells at each division.

Previously Balbiani (1885) had succeeded in tracing the germ-cells ("pole-cells") of the dipter *Chironomus* to an early stage of differentiation in the segmenting egg. "Pole-cells" were first reported by Robin in 1862 for certain other Diptera, and subsequently (1863) also by Weismann for *Chironomus*. Hasper has quite recently (1911) identified one of the first four cleavage-cells as the progenitor of all the germ-cells in *Chironomus*.

A similar history was traced by Häcker for the germ-cells in *Cyclops;* here the germ-cell progenitor is recognizable at the 32-cell stage.

Hegner (1909, 1914, 1915) has worked out very completely the early germ-cell path ("Keimbahn") in certain Diptera and in chrysomelid beetles, where the diagnostic marks are a polar differential granular cytoplasmic content, the "pole-disk," constituting a germ-cell determinant. In the dipter *Miastor*, Hegner (1914) describes a segregation

of the germ-cells at the 8-cell stage, in a number of other cases at the 32-cell stage.

The literature touching the germinal path ("Keimbahn") in invertebrates is fully and very ably reviewed by Hegner in the Journal of Morphology (vol. 20, p. 231; vol. 25, p. 375; and vol. 26, p. 495). The reader is referred to these papers for bibliographic lists.

GERM-CELL ORIGIN IN VERTEBRATES.

The detailed and critical study of the origin of the germ-cells in vertebrates may be said to have begun with the observations of Hoffman (1893) on certain birds (including *Gallinula chloropus*, *Sterna paradisea*, and *Hæmotopus ostralegus*), which disclosed primordial germ-cells in the entoderm and splanchnic mesoderm far removed from the site of the future gonads, and before a "germinal epithelium" had developed (23-somite stage).

The work of Eigenmann (1892, 1897) marked an epoch in this line of investigation. He clearly demonstrated the extra-regional origin of primordial germ-cells in the viviparous teleost *Cymatogaster* and showed that they closely approximated the size and appearance of the fifth-cleavage blastomeres, on the basis of which he argued that the germ-cells were segregated at the fifth-cleavage stage. An extra-regional entodermal origin of primordial germ-cells has been described also by Dodds (1910) in the teleost *Lophius*.

In 1904 D'Hollander described the origin of the germ-cells (oögonia) in the chick embryo of the tenth day from epithelial buds of the peritoneal epithelium, in conformity with the earlier views of Waldeyer. This discrepant observation is now elucidated by the recent works of Swift (1915, 1916), which show that such buds do indeed occur, but that they *include*, but do not *originate*, primordial germ-cells. The latter have an extra-regional origin.

Beard in 1904 described the extra-regional origin of primordial germ-cells also in the skate *Raja batis*. These cells are said to arise in the anterior portion of the embryonic shield and to migrate along a very definite route to the gut entoderm, thence through the splanchnic mesoderm to the future gonad. A very similar later history has been described by Woods (1902) also for the dog-fish, *Squalus acanthias*.

Beard and Eigenmann agree that the germ-cells do not divide for a long period in the early development of the embryo, that the original number remains constant except for a slight diminution due to degeneration, and that a few may become stranded in the migratory process in locations outside of the genital glands.

The most detailed and satisfactory study of the germ-cells in the chick has been made by Swift (1914, 1915, 1916) with the aid of mitochondrial technics. He describes the origin of the primordial germ-cells in a crescentic antero-lateral area from germ-wall entoderm

at the margin of the area pellucida, during the primitive-streak stage and until 3 somites are formed (23-hour stage). The cells by amœboid activity enter the blood-spaces of the mesoderm, which subsequently grows into this portion of the primitive proamniotic area, and are then carried to all parts of the embryo and the vascular area. The germ-cells remain widely distributed in this way until about the time when the embryo has 20 somites. Numbers of those in vascular channels are said to undergo mitotic division, while some suffer degeneration. The germ-cells remain scattered throughout the blood-vessels until about the 22-somite (44-hour) stage, only a few cells appearing in the splanchnic mesoderm. At the 23 to 25 somite (50-hour) stages the majority of the germ-cells have escaped from the blood-vessels and are now found almost exclusively in the tissue of the splanchnic mesoderm near the cœlomic angle. After the 25-somite stage no germ-cells remain in the blood-vessels. In embryos of from 30 to 33 somites (60 to 72 hours) the primordial germ-cells have migrated to the root of the mesentery and into the cœlomic epithelium on both sides of the cœlomic angle. Here they remain until the formation of the gonad begins, when they gradually pass into that organ and become the oögonia and the spermogonia of the ovary and testes respectively.

Swift describes also a very prominent attraction-sphere, persisting without change from the origin of the germ-cell to its entrance into the gonad, and he uses this mark as the chief character for differentiating between germ-cells and adjacent elements. Such a uniform and conspicuous criterion was lacking in the loggerhead-turtle embryo and could not be employed in the identification of the germ-cells.

Others of the more recent workers with chick embryos include Nussbaum (1901), Rubaschkin (1907), and von Berenberg-Gossler (1912). These investigators agree in finding primordial germ-cells in the entoderm and splanchnic mesoderm lateral to the cœlomic angle in embryos of about 22 somites. In a more recent paper von Berenberg-Gossler (1915) confirms Swift in his observations that primordial germ-cells appear within the vascular channels in chick embryos prior to the 25-somite stage.

Firket (1914) maintains that in birds the majority of the primordial germ-cells disintegrate, though some may possibly come to "maturity" in the wall of the gut; and he views their extra-regional appearance as of only phylogenetic significance, reminiscent of the definitive sex-cells of lower vertebrates.

The work of Allen on the turtle *Chrysemys marginata* (1906, 1907) and on the ganoid fishes *Amia* and *Lepidosteus* (1911) has greatly advanced our knowledge regarding the origin and development of the primordial germ-cells in vertebrates. In these three forms Allen has likewise demonstrated an extra-regional entodermal origin. In *Chrysemys* the germ-cells ("sex-cells," Allen) are described as arising

from the entoderm near the lateral margin of the area pellucida at a level beginning about the cephalic extremity of the pronephros and extending on either side to a point just behind the caudal tip of the embryo. The total number of the original quota of germ-cells is said to vary from 302 to 1,744, with an average of about 1,100 (Allen, 1907). No mitoses were observed in these cells until the 10-mm. stage of development. During the migration of the sex-cells many are said to lag hopelessly behind, while others may go entirely astray. Upon an average 47.7 per cent are estimated to finally reach the sex-glands, while the remainder are said to come to rest in the alimentary tract, the mesentery, and the region between the root of the mesentery, the aorta, and the mesonephroi.

It is important to note that Dustin (1910) records a much smaller average number of germ-cells in *Chrysemys* embryos of this stage, the extremes being given as 158 and 415. This number is much closer to my own count (see chart, page 323) for *Caretta*.

In *Lepidosteus* the primordial germ-cells have a very similar origin. They are described as migratory to a high degree. In *Amia* they are said to arise from the entoderm of the roof and margin of the floor of the subgerminal cavity.

The history of the germ-cells in *Chrysemys* is practically identical with that above described for *Caretta*. However, I find no evidence in *Caretta* to support Allen's conclusion that "large numbers" of cells fail to migrate eventually to the gonads, but undergo degeneration in these extra-regional locations. Rarity of actual degenerating cells, or persisting representatives of any kind in later stages, indicates an ultimate migration of at least the vast majority of the extra-regional germ-cells into the genital-glands. Moreover, an occasional germ-cell may be seen dividing during all the earlier stages.

Dustin (1907) describes the origin of the sex-cells ("gonocytes") in a 3 mm. *Triton* larva from the medial portions of the lateral plates of mesoderm in the caudal half of the body. These original sex-cell masses become approximated medially and subsequently fuse to form a longitudinal rod of sex-cells just above the dorsal root of the mesentery. At about the 14 mm. stage the majority of the original sex-cells are said to degenerate and disappear, and a new generation to be differentiated from the peritoneal epithelium of the genital ridge. A similar mode of origin and history of the germ-cells is described for *Rana* and *Bufo*. As regards *Rana pipiens*, Allen (1907) describes a dorso-medial migration of germ-cells from the gut entoderm at the time when the two lateral plates are approximated in the formation of the mesentery. The initial mesodermal origin described by Dustin is accordingly here an illusion.

In the matter of an entodermal origin of the germ-cells in *Rana*, Allen has been confirmed by Kuschakewitsch (1908), and by King

(1908) as respects *Bufo lentiginosus*. Miss King finds no evidence in support of a secondary source of germ-cells from the peritoneal epithelium. Moreover, in *Chrysemys*, Allen (1906) traced the germ-cells to maturity without finding any evidence of a transformation of peritoneal cells into germ-cells. The same is true also of *Caretta* at least to the 32-day stage.

Dustin (1910) has repeated the investigation of Allen on *Chrysemys marginata*, and now confirms Allen's observations regarding the entodermal origin of the extra-regional germ-cells in this form. Dustin still claims, however, that in this form also there is a secondary peritoneal source of origin of germ-cells or "gonocytes." But his illustrations do not seem to support this conclusion. The so-called "secondary gonocytes" as illustrated are smaller than certain other germ-cells designated "primary gonocytes," but no unequivocal transitions appear between the mesenchyme cells of the sexual gland or its peritoneal cells and the secondary gonocytes.

In a postscript (p. 33) Allen (1911) records preliminary results in the case of *Necturus* and *Amblystoma* similar to those reported by Dustin for *Triton*, namely, an actual mesodermal origin of sex-cells. Allen disposes of the conflicting views regarding the origin of germ-cells in amphibia by the claim that in the urodeles the usual source of origin of the germ-cells is the mesoderm; that in the anurans the source of origin is the entoderm; at the same time, he admits the possibility of exceptions.

A similar mesodermal origin of the sex-cell is described also for the teleost *Lophius* (Dodds, 1910). Wheeler's results on *Petromyzon* (1899) indicate that the germ-cells may become included in the mesoderm at the time it separates from the entoderm. Jarvis (1908) reports an entodermal origin of the primordial germ-cells also in the horned toad, *Phrynosoma cornutum*.

Gatenby (1916) has reexamined the evidence in the case of *Rana* (*temporaria*), *Salamandra*, *Triton* (*molge*), and *Amblystoma*. The observations on these forms accord in all the main points and "resemble *Bufo* in the history of germ-cell production," which it is claimed proceeds by a differentiation of germ-cells ("secondary gonocytes") from the germinal epithelium, in accord with Waldeyer's original theory. Gatenby thus disagrees with the conclusions of King (1908) for *Bufo lentiginosus*, Allen (1907) for *Rana pipiens*, and Kuschakewitsch for *Rana esculenta;* but agrees with the conclusion of Dustin (1907) for *Triton*, *Rana*, and *Bufo*, and with the earlier conclusion of Kuschakewitsch (1907) for *Rana pipiens*.

Gatenby, however, admits "that some germ-cells possibly migrate from the entoderm of the yolk-sac," but "feels sure that during life very large additional reinforcements of germ-cells arise in the epithelium of the gonad of Amphibia" (p. 276). His criticism, then, of the

work of King, Allen, Beard, and others who hold to the view of a genetic independence between cœlomic epithelium and germ-cells is that they have studied only the earlier periods of the ontogeny. But in the male chick Swift has now (1916) examined also the young post-incubation stages, as late as 10 days, and still finds all the evidence in accord with the segregation view.

Gatenby describes the tadpole ovary as formed of (1) germ-cells of peritoneal origin; (2) germ-cells of retro-peritoneal origin; (3) germ-cells of endoderm (yolk-sac) origin. He inclines to believe that the germ-cells of the yolk-sac are all laid at the first spawning. He describes and illustrates intermediate stages between peritoneal cells and young germ-cells, but one is forced to the criticism that his figures give no adequate support to his claim of a transformation of mesoderm-cells into germ-cells.

In the lizard embryo (*Lacerta agilis*), according to von Berenberg-Gossler, the migration of the "primordial germ-cells" is not so strongly limited to the early developmental stages as described, *e. g.*, for *Chrysemys;* and the cells ("entodermal wandering cells") are relatively much more numerous; nor do any of them disintegrate; they become converted into mesoderm-cells. "The migration of cells out of the entoderm in *Lacerta* has no other significance than a dilatory development of mesoderm from entoderm" (pp. 247–248). He extends this interpretation to cover the so-called extra-regional genital cells of reptiles and mammals. As mesodermal cells they may possibly metamorphose into cells of the cœlomic epithelium, as earlier maintained by Hoffman. They wander from the entoderm largely to the location of the future pronephric (Wolffian) duct, to the construction of the caudal portion of which they greatly contribute. Occasional cells are found also in the somatopleure. None of the cells while within the mesoderm were found in process of mitosis. All these cells are believed to become changed into mesoderm-cells; thus they apparently disappear, but from some of those scattered among the cells of the peritoneal epithelium definitive germ-cells arise. This interpretation eases the difficulty of accepting the view of certain investigators (*e. g.*, Firket, Dustin, Gatenby, Felix) that both primary and secondary genital cells are formed, the primary becoming displaced through "degeneration" by the secondary. The whole genital gland would then arise from mesoderm, as maintained by some of the earlier investigators.

No attempt is made by von Berenberg-Gossler to apply the above interpretation to conditions in the chick, where these cells during early stages have an intravascular distribution. However, he expresses doubt regarding the germ-cell nature of these intravascular "germ-cells," many of which are in process of degeneration. A common interpretation of the phenomena relating to these "entodermal wan-

dering cells" or "primordial germ-cells" in the lizard and the chick is obviously difficult if not actually impossible on this basis.

Rubaschkin (1908, 1909) describes an entodermal origin of primordial germ-cells also in rabbit and guinea-pig embryos; and he traces a migration route practically identical with that here described for *Caretta* and that described for *Chrysemys* by Allen. In the cat embryo, Winiwarter and Sainmont (1909) describe similar sex-cells in the embryonic ovary, but they interpret these cells as hypertrophied forms of the ordinary cells of the sex-cords.

Nagel (1889) described extra-regional germ-cells also in the human embryo, located near the pronephric duct. In 1911, Felix likewise described them in human embryos. In 1912, Fuss published his results of a study of 3 human embryos (of 2, 3, and 4 weeks) and 17 other mammalian embryos, including rabbit embryos and pig embryos (of 7 to 14 mm.). In the human embryo the sex-cells are said to be capable of amœboid motion; this movement is combined with a passive migration, the result of growth in the enveloping tissues. Fuss declares that aside from the germinal path there are no germ-cells, and that the so-called "germinal epithelium" plays no large rôle in the origin of germ-cells in mammals. The germ-cells are said to be present long before the differentiation of the sexual gland takes place. They arise from the entoderm, and after closure of the gut they migrate through the mesentery to the genital gland—in the human embryo about the fourth week, in the rabbit embryo about the thirteenth day, and in the pig embryo of about 14 mm. On entrance into the gland they may undergo active mitotic proliferation.

DISCUSSION AND CONCLUSIONS.

It would seem that the evidence is now sufficient to amply support the hypothesis of the extra-regional origin of the germ-cells first clearly enunciated by Nussbaum (1880) in opposition to the teaching of Waldeyer (1870), who derived the germ-cells by process of differentiation from the peritoneal epithelium. Swift's most recent publications (1915, 1916) clear also the additional obscurity regarding the germ-cells produced by Felix, who claimed that the primordial germ-cells ("primary genital cells") in amniotes, including man, have an extra-regional origin, but later disappear and become replaced by "secondary genital cells," the definitive germ-cells, differentiation products of the peritoneal epithelium. This is also the view of Dustin regarding certain amphibia and *Chrysemys*, and of Gatenby for the frog. Swift has shown for the chick that the sex-cords arise as ingrowths of proliferating regions of peritoneal epithelium into the subjacent mesenchyma of the genital fold (beginning about the sixth day of incubation), and that the sex-cells which have migrated from extra-regional areas to the peritoneal epithelium become involved in

the sex-cords, where as oögonia and spermogonia they undergo proliferation and further differentiation.

Considering all the forms studied, from *Ascaris* to man, the evidence is all but complete for a morphologic continuity of the germ-cells, a "Keimbahn," or germinal path. According to Buchner (1910) no evidence appears for such a germinal path in annelids, mollusks, and echinoderms, nor according to Fick (1906) for plants. But these are simply instances regarding which our evidence is as yet very incomplete.

The evidence derived from this study of the embryo of the loggerhead turtle supports the theory of extra-regional origin of germ-cells and, so far as it goes, the Keimbahn theory. The facts are most closely in line with those reported by Allen for *Chrysemys*, by Woods for the dog-fish, and by Beard for the skate.

This work had in view three chief objects: (1) to discover the origin of the germ-cells and the route and manner of possible migration; (2) a careful examination of the cellular contents of all blood-channels with the expectation of removing a possible confusion between germ-cells and certain blood-cells; (3) to find a basis for harmonizing the results of von Berenberg-Gossler in the case of *Lacerta* with those reported for other reptiles, and possibly other vertebrate forms.

My first surprise was to find the facts in *Caretta* closely like those described by Allen for *Chrysemys*. The germ-cells are segregated in the entoderm from about the second to fifth day near the lateral border of the area pellucida, from a point near the cephalic tip of the pronephros to the caudal extremity. Up to the fourth day occasional cells occur also in the area opaca near the border of the area pellucida. This indicates the first step in the migration, namely, from area opaca to lateral border of area pellucida caudally; since germ-cells disappear later from the area opaca. In the area pellucida the germ-cells then become fairly closely aggregated into two bilateral cords. These are, however, not of uniform thickness throughout, but have an interrupted or segmental character, suggesting "gonotomes."

At about the fifth day germ-cells wander into the splanchnic mesoderm and migrate medially. When the hind-gut becomes closed (from the sixth to the tenth day) germ-cells become included in its mucosa and in the more peripheral portion of wall. From here many migrate through the mesentery across the angle of the cœlom to the differentiating sex-gland at the seventh day, a few apparently degenerating within the gut-wall. At the thirty-second day they are distributed among the peritoneal epithelium of the developing sex-gland and in the subjacent mesenchyma. Only very occasionally is a cell found extra-regionally at this stage, and apparently no sex-cords have as yet begun to form; but neither is there the slightest evidence of a differentiation of the so-called germinal epithelium into genital cells. In the earlier stages a few germ-cells may be seen in mitosis and an occasional cell may apparently divide at any stage.

My second surprise was to find practically no germ-cells in any vascular channels; only two exceptions were noted. Nor does there seem to be any possibility of confusion of sex-cells with blood-cells, judging from Swift's figures or on the basis of my sections of the loggerhead-turtle embryo. Swift's observations have now been confirmed by von Berenberg-Gossler for chick and duck. Von Berenberg-Gossler was unable, however, to find similar conditions in the case of the lizard embryo. The condition of abundant intravascular germ-cells in the chick embryo and the duck embryo is so far unique. Conditions respecting the germ-cell history in birds are evidently different from those obtaining in reptiles and certain other amniotes, and depend upon the cephalic (pro-amniotic) site of origin of the germ-cells and their close relation to the vascularizing mesoderm.

Considering the initial very close relationship (both spatial and genetic) between the entoderm and the mesoderm at the time when the latter is in the early stages of vascularization and the considerable migratory capacity of the primordial germ-cells, the presence of these cells outside of the usual migration route, even in the blood-vessels, is nothing extraordinary. In the chick the initial source of origin is in the entoderm at the anterior extremity of the blastoderm in a region originally free of mesoderm. The cells begin their first migration about the time the vascularizing mesoderm invades this region, and so readily enter into the blood-vessels by which they are transported in large part caudally to the medial splanchnopleure, whence they pass via the mesentery to the gonads (Swift). In forms like the turtle and the dog-fish, where the germ-cells are originally scattered caudally in the area opaca, they do not in large numbers come into so intimate early relation with the young blood-vessels, and in consequence reach the medial splanchnopleure largely via the visceral layer of the mesoderm by their own amœboid activity, meanwhile passing through a temporary stage of sharp segregation into the paired cords of the area pellucida. But even here it is apparently an easy matter for the germ-cells to become involved with the blood-channels either through active migration or passive inclusion. There is nothing, therefore, inherently contradictory or unique in the condition of the germ-cell migration as it obtains in the chick and duck. The difference is one of degree rather than kind, and depends upon the difference in initial location of the primordial germ-cells with respect to the mesoderm and the head end of the embryo.

The very careful work of Allen, in which he counted the number of germ-cells at the different stages of development in different areas in *Chrysemys*, clearly shows that the total number of germ-cells in different individuals of a species varies within wide limits (302 to 1,744), and that the time and rate of migration also vary considerably. I therefore made no attempt to approach the question of the route and manner

of migration on the basis of germ-cell counts. The fact that at progressively later stages in development the germ-cells were found in the entoderm near the lateral border of the area pellucida, then in the visceral plate of the lateral mesoderm and more medially in the entoderm (later also in the closed gut and enveloping mesenchyma), then in the mesentery, and finally across the cœlomic angle among the peritoneal epithelium of the sexual gland (genital fold), indicates that this represented the actual route of migration. The further facts that only an occasional germ-cell was seen in mitosis throughout the series (second to thirty-second day), that only a few were seen in conspicuous stages of degeneration, and that practically none appeared in blood-vessels (and only a few in the periaortic mesenchyma) seems to prove the indicated route as the actual and substantially exclusive migration path. In the case of *Cymatogaster*, also, Eigenmann found that the sex-cells do not divide from the period before the first somites have formed—at which time they are first distinguishable—up to about the period when the young fish reaches a length of 7 mm.

The close similarity, which in early stages amounts practically to an identity, between the primordial germ-cells and the cells of the entoderm (especially the yolk-sac entoderm) need not, and almost certainly does not, imply a derivation of germ-cells from entoderm-cells. In the first place, the germ-cells can always be distinguished from the entodermal cells (in spite of structural and tinctorial similarity between the cells) by the rounded (spherical or oval) form of the germ-cells. The cells of the yolk-sac entoderm, which they resemble most closely, are of irregular shape. Occasionally a germ-cell may become wedged in between the entoderm-cells and so forced into very intimate cytoplasmic relationship and into irregular form, in which condition it may be impossible to distinguish between the two; but the resemblance signifies simply, most probably, a similar low grade of differentiation from the original blastomeres. The germ-cells are therefore not *derived* from the entoderm-cells by differentiation. These two types of cells are similar because they are practically similarly undifferentiated, both containing a large, finely granular, pale nucleus and a large cytoplasmic content of yolk-globules. The germ-cells simply remain *dispersed* among the entodermal-cells after the segmentation stages and from here migrate via the area pellucida to the mesentery and thence to the gonads. The germ-cells here, as in certain invertebrates (*e. g.*, *Ascaris*, Boveri) most probably have been held apart from the soma-cells since an early segmentation stage and then, from widely scattered areas, have migrated through progressively more sharply segregated foci to the gonads, maintaining thus a continuous germ-cell cycle from blastomeres (perhaps a particular portion of the fertilized egg, as, for example, the pole disk of certain Diptera, Hegner) to the gonocytes of the definitive genital glands.

It remains to discuss the mode of the extravascular migration—*i. e.*, whether active (amœboid) or passive, or both. A passive migration is strongly urged by von Berenberg-Gossler. He thinks the germ-cells are passed from the lateral to the medial entoderm, and from the lateral visceral mesoderm to the mesentery, and then across the cœlomic angle by the mechanical process of unequal growth. Growth is no doubt a factor in the shifting. But the chief factors inhere in the cells themselves, which (judged from their shape and the manner in which the mesenchymal cells are compressed in the direction of a path towards the root of the mesentery) is an active amœboid movement of the cells. The cells evidently move medially both within the endoderm and in the mesoderm. Such as lie medially in the entoderm become inclosed within the hind-gut, whence the majority later migrate into the mesentery.

Only two germ-cells were found at any stage within any blood-channels. But no confusion between blood-cells (hemoblasts) and germ-cells seems possible. The germ-cells are at all stages larger than the largest granulocyte (eosinophil, whose granules also stain black in iron-hematoxylin) and the largest hemoblast, the character and staining capacity of whose nucleus is very different. There is therefore no room for doubt that in chick (Swift) and duck (von Berenberg-Gossler) the germ-cells actually do migrate (passively) towards the genital ridge largely through the blood-vessels. The locus of original segregation in birds is such as to provide in the blood-channels the most favorable method for the longer portion of the route of migration to the sexual gland.

As Allen has already suggested, the reason for the mesodermal origin of the germ-cells in urodeles and possibly certain other vertebrates may be a relatively early separation of mesoderm from entoderm—that is, the germ-cells of the primitive entoderm may have become separated with the portion which contributed to the splanchnic layer of the mesoderm before they could migrate from the entoderm proper.

In *Lacerta* von Berenberg-Gossler describes cells comparable to the so-called primordial germ-cells passing from the entoderm to both layers of the mesoderm, and to the dorsal layer of the intermediate cell-mass, where caudally the cells are said to contribute largely to the formation of the Wolffian duct. Von Berenberg-Gossler interprets his results to mean a belated origin of mesoderm from entoderm. He very ingeniously suggests that these "germ-cells" wander also to the sex-gland, where they may transform into mesenchymal-cells, and then retransform into germ-cells, but presents no histologic data in support. Von Berenberg-Gossler's results and his interpretation are unique and do not seem to be capable of being brought into harmony with the results obtained in any other form thus far studied, nor with any common interpretation. Von Berenberg-Gossler's interpretation is

more or less plausible, but can not be said to be wholly satisfactory. The condition in the lizard may be simply an exaggeration of that in other forms where a certain number of germ-cells go astray during the period of migration.

Since germ-cells can migrate by their own activity, it is not difficult to conceive of their presence anywhere, even in the ectoderm, and certainly not in the developing Wolffian duct, where, unless they retrace their course towards the genital gland, they may undergo degeneration (or possibly lie dormant until a favorable influence may excite to the production of a tumor or a teratoma), but that they should contribute to the formation of the Wolffian duct is thus far an anomalous phenomenon.

When one considers in common conditions in the chick, lizard, toads and frogs, salamanders, and turtles, it becomes clear that there is great variation in the manner in which the comparable elements (the "primordial germ-cells") arise, migrate to the sexual gland, and develop. Underlying these discrepant phenomena, however, there appears a fundamental harmony, namely, an early segregation of germ-cells from somatic cells, a germinal path from blastomeres to genital cells of the genital gland, probably from blastomeres to sperm and ova; a migration from the entoderm (or its derivative, the mesoderm, in urodeles and lamprey), through the splanchnic layer of the mesoderm to the mesentery and thence to the gonads (with possible aberrant migrants anywhere).

And in the light of Swift's latest researches in the case of the sex-cords and the ova of the chick ovary and the spermatozoa of the testis, it now seems clear (notwithstanding the contradictory findings of Dustin in *Chrysemys* and of Gatenby in the frog) that the definitive germ-cells have had an extra-regional origin and do not arise by differentiation from germinal epithelium (cœlomic epithelium), but become involved in columnar invaginations of these cells to form sex-cords. Certain observations (*e. g.*, those of Felix, Dustin, and Firket) to the effect that the primordial germ-cells ("primary genital cells") degenerate early and are replaced by cœlomic derivatives in the genital gland, may possibly be explained by the facts that in certain amniotic forms the primordial germ-cells degenerate relatively more extensively and that mitosis does not generally appear until the remainder reach the sex-glands, where it becomes active in those cells involved in the sex-cords formed by the "germinal epithelium." The great proliferative activity of the remaining primordial germ-cells in the formation of oögonia and the spermogonia, among the cells of the cœlomic epithelium, gives the appearance of an active differentiation and derivation from the cœlomic epithelial-cells. The products of this intense proliferation are relatively smaller than the earlier germ-cell progenitors, which phenomenon makes the superficial resemblance between growing cœlomic epi-

thelial cells and the definitive germ-cells more close. At any rate, no clear case has yet been made out for the differentiation of germ-cells from cœlomic epithelium at any stage in any form.

There is no evidence in *Caretta* of a differential mitochondrial content between the germ-cells and somatic cells, as maintained by Tschaschin in the case of the chick embryo and by Rubaschkin in the case of certain mammalian embryos; and as denied by Swift and von Berenberg-Gossler. The germ-cells are distinguished from the somatic cells by their larger size, their generous yolk-content, their large vesicular oxyphilic nucleus, with its delicate, finely granular, radially arranged, nuclear network. These nuclei resemble more closely those of the entoderm-cells of the area opaca, indicating a low grade of differentiation of these two cells and a close genetic relationship inhering most probably in a common near ancestor, from which they have departed but little in their slight progressive differentiation. This indicates also that the germ-cell commonly remains undifferentiated until a relatively late period in its history, when it takes residence in the genital gland. Its load of nutritive material in the form of yolk (still present in the 25-day stage) also indicates a low grade of differentiation and suggests the cause of its lack of proliferative capacity until digestion of this yolk is completed, and the further reason of its great capacity for later proliferation and differentiation, especially in the male.

But many more researches covering the later periods of the history of the ovary with special reference to the so-called "germinal epithelium," such as those of Swift on the chick and of Gatenby on the frog, are needed before the hypothesis of a germinal path for vertebrates can be said to be completely established. In view of conditions in the gonads of certain annelids, mollusks, and echinoderms, where enormous numbers of germ-cells are formed during successive years, and the disagreements which still exist with respect to such relatively simple forms as frog (Allen and King *vs.* Dustin and Gatenby), chick (Swift *vs.* Firket), and the turtle, *Chrysemys* (Allen *vs.* Dustin), the claims that two series of genital cells occur—a primordial and a secondary or definitive—the one derived directly from a blastomere of the later cleavage stages and so segregated from the somatic cells, and the other derived by differentiation from the cœlomic epithelium of the genital ridge, and so modified soma-cells, while on logical grounds inherently improbable, and without firm observational basis—and disproved on histologic grounds, I believe, in the case of *Caretta*—can not perhaps be said to have been definitely disposed of.

SUMMARY OF RESULTS.

1. Twenty-five embryos of the loggerhead turtle (*Caretta caretta*), ranging from the second day (5 somites, 2 mm. length) to the thirty-second day of incubation, were employed in this investigation. The early history of the primordial germ-cells is very similar to that described by Allen for the turtle *Chrysemys* and by Woods for the dog-fish.

2. The primordial germ-cells migrate during the second day from the yolk-sac entoderm, where they were more or less widely scattered caudally, into the lateral border of the area pellucida on each side of the embryonic disk. Here they become sharply segregated by the beginning of the third day into two bilateral cords situated in the entoderm of the area pellucida laterally in the caudal half of the disk. In the 2-day embryo they extend from the neurenteric canal to the end of the primitive streak; in the 3-day embryo from the sixth somite to the caudal extremity of the streak. The cords become more medially placed, make a linear connection with the overlying visceral mesoderm, and their cells migrate during the fifth day into this mesoderm, and thence medially (during the sixth and seventh days) towards the root of the forming mesentery of the closing hind-gut. Individual cells migrate medially also within, or back into, the entoderm of the gut. The germ-cells in the medial entoderm become included in the mucosa of the closed hind-gut, those in the mesoderm in the enveloping mesenchyma and the gut end of the mesentery. From these locations the majority of the germ-cells subsequently (seventh to twelfth day) migrate up the mesentery and across the cœlomic angle into the future sexual gland. They become incorporated among the mesenchymal cells of the gland and the covering peritoneal epithelium, where they suffer no striking change in form, size, or content at least as late as the thirty-second day of incubation.

3. The germ-cells migrate by amœboid activity, assisted in small part probably by the factor of unequal growth, involving the shifting of the medial portion of the splanchnopleure to the mesentery and the dorsal portion of the mesentery to the gonads.

4. The migration period is not sharply limited. It is at its height from the seventh to the twelfth day, and practically ceases about the sixteenth day. But occasional extra-regional cells may still be found in the gut and mesentery at the 32-day stage—usually, however, showing signs of degeneration.

5. A certain number of germ-cells migrate out of the regular germ-cell route and go astray. Such strays are especially numerous in the periaortic mesenchyma caudally, where they may become incorporated among the neuroblasts of the developing peripheral sympathetic ganglia. The majority of these strays probably degenerate *in situ*, but some may possibly persist to form, under the proper pathologic

stimulus, a focus of neoplastic growth. An occasional cell is found also in the blood-vessels of this region. Such may be carried by the blood-stream to distant regions and perhaps again enter the mesenchyma or degenerate within the vessels.

6. The total number of primordial germ-cells counted in a 12-day embryo is 352, the number within the gonads being about equally divided between the two (118 left to 127 right).

7. Occasional cells may divide by mitosis, or undergo degeneration, at any stage of their history or at any point of the route. Mitoses are more numerous during earlier stages and among the entodermal cells; degeneration is more general during the later stages and in the mesenchyma of the closed hind-gut.

8. No germ-cells were found contributing to the formation of the Wolffian duct. There is no evidence in this form in support of von Berenberg-Gossler's claim, on the basis of his observations on the lizard embryo, that the so-called primordial germ-cells represent simply a belated stage of mesoderm formation from entoderm.

9. The germ-cells do not differ from young somatic cells in the character of their mitochondrial content. The mitochondria include granular as well as beaded-rod and filamentous forms.

10. No transition stages between cœlomic epithelial cells and germ-cells appear up to the 32-day stage. From the 16-day stage on, when the nuclei of some of the germ-cells within the gonads become coarsely granular and the reticulum stains more deeply, apparent transition stages occur between the larger of the mesenchymal cells and the smaller included subepithelial germ-cells. But no secure histologic basis can here be found for separating the germ-cells of the gonads into large "primary genital cells" and smaller "secondary genital cells" (Felix) or "gonocytes" (Dustin) derived by process of differentiation from the cells of the "germinal" (peritoneal) epithelium or the subjacent mesenchyma. The size variations among the germ-cells of the gonads of the older stages are no greater than in the original cords of the area pellucida or in the subsequent early stages; and the cytologic similarity between the two dimensional grades of cells is much closer than between the larger mesenchymal cells and the smaller germ-cells.

11. The evidence derived from a study of the *Caretta* embryos is in complete harmony with the idea of a single uninterrupted line of sex-cells from primordial germ-cells to oögonia and spermogonia, and with the hypothesis of a vertebrate Keimbahn or continuous germinal path.

12. The variations in the distribution of the primordial germ-cells during earlier embryonic stages described by various investigators for a number of vertebrate forms—as pertains both to their presence in blood-vessels (chick, Swift; duck, von Berenberg-Gossler) and in various regions and tissue remote from the more direct and more usual germinal route (Wolffian duct and somatopleure in the lizard,

von Berenberg-Gossler; and sympathetic ganglia in the loggerhead turtle, Jordan); and to their apparent primary (urodeles) or secondary (anurans and other vertebrates) derivation from the splanchnic layer of the lateral mesoderm—are incidental to their original location with respect to the embryonic area and the vascularizing mesoblast of the blastoderm, and to their amœboid capacity. Since the primordial germ-cells are genetically directly related to neither of the secondary germ-layers, their origin in either (entoderm or mesoderm) has no fundamental significance. Since they are capable of amœboid activity, and may become included in blood-vessels, they may migrate anywhere, and so occur in any location, from where they may subsequently migrate again to the more direct germinal path, or perhaps disintegrate. The fact of fundamental significance with respect to the primordial germ-cells is their original extra-regional distribution and their direct genetic independence of the soma-cells.

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DESCRIPTION OF ILLUSTRATIONS.

FIG. 1.—Composite drawing of left side of mesentery near its root in the 11-day embryo. All the cells shown are from the immediate vicinity of the same section. *M*, mesothelium of mesentery; *h* and *e*, hemoblast and erythrocyte, respectively, in the aorta; *b*, eosinophil blood granulocyte, lying among the mesenchymal cells; *a*, typical primordial germ-cells (primary genital cells), laden with yolk-globules; *i*, attraction sphere, shown both in the granulocyte and the genital cell. Similar germ-cells appear also in this and immediate sections in the gut (both among the entodermal cells and in the enveloping mesenchyma) and among the mesothelial cells of both the mesentery and the genital ridge. This section is very similar to that of figure 2 (plate V). Flemming fixation, iron-hematoxylin stain; magnified 1,000 diameters.

FIG. 2.—Portion of genital ridge of same 11-day embryo, showing a germ-cell among the flattened cells of the peritoneal epithelium; the germ-cell contains many mitochondria, of granular, rod, beaded-rod, and a few of filamentous forms; also some yolk-granules. *b*, an eosinophil granulocyte. ×1,000.

FIG. 3.—Section through cœlomic angle of same 11-day embryo, showing a germ-cell among the peritoneal epithelial-cells of both the mesentery (*aa*) and the genital ridge (*ac*); the germ-cell *ab* lies among the mesenchymal cells of the mesentery, apparently in process of migration into the mesothelial layer. Both *aa* and *ac* also are in amœboid activity. Cell *ac* is loaded with an apparently continuous mass of yolk material. Magnified 1,000 diameters.

FIG. 4.—Germ-cells from the 10-day embryo (similar to fig. 2, plate V), in the region of the hind-gut. (Helly fixation, Giemsa stain.) *a*, germ-cell from the entoderm of the gut (the nucleolus stains a deep violet, the nucleus, with its granular reticulum stains a light bluish-pink, and the reticular vacuolated cytoplasm stains a pink color); *b*, germ-cell in mesenchymal layer of gut; *c*, germ-cell in the peritoneal epithelium of the genital ridge; *d* and *e*, germ-cells from the mesenchyma of the developing genital gland; *f*, eosinophil from mesentery (granules deep red, nucleolus blue). ×1,000.

FIG. 5.—Erythroblast within a blood-vessel, in the 11-day embryo. ×1,000.

FIG. 6.—Germ-cells from 12-day embryo. (Helly fixation; iron-hematoxylin stain.) *a*, germ-cell from among the entodermal cells of the still open portion of the hind-gut (fig. 1, plate VI); *i*, attraction sphere; *b*, germ-cell from among the peritoneal epithelium of the genital ridge (the nucleolus is stained a deep black, the granular nucleus a brownish gray, the vacuolated cytoplasm a light gray); *c*, germ-cell from middle of mesentery closely enveloped by flattened mesenchymal cells, simulating a close-fitting capsule. ×1,000.

FIG. 7.—Germ-cell from "germinal epithelium" of genital-gland of 22-day embryo. ×1,500. Helly fixation; stained *in toto* with Delafield's hematoxylin. The nucleolus stains a deep blue, the granular nucleus light blue, and the reticluar cytoplasm a still lighter blue.

FIG. 8.—Germ-cell from subepithelial mesenchyma of genital gland of 25-day embryo. The cell is closely enveloped by flattened mesenchymal cells, simulating a capsule. ×1,500.

FIG. 9.—Germ-cell from submesothelial mesenchyma of sexual gland of 32-day embryo. ×1,500.

FIG. 10.—Germ-cells and blood-cells from the 8-day embryo. *a*, germ-cell from the splanchnic layer of lateral plate of mesoderm, near the border of the area pellucida; *b*, germ-cell from mesentery closely enveloped by flattened mesenchymal cells; *c* and *d*, hemoblasts from mesochyma of mesentery and from the aorta respectively. ×1,500.

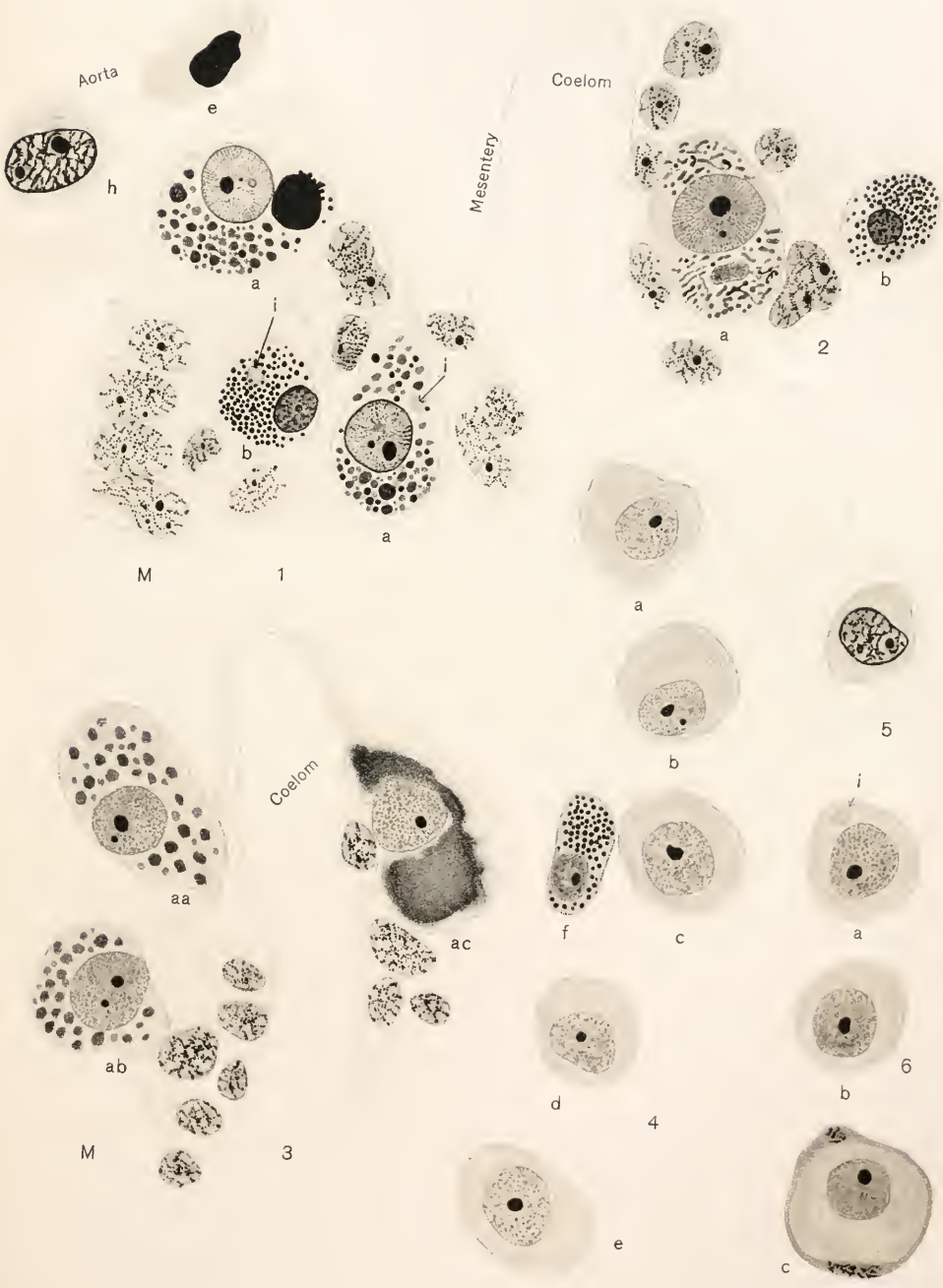
FIG. 11.—Section of entoderm of open gut of 7-day embryo from near the border of the area pellucida, showing a germ-cell among the entodermal cells. The cytoplasm of the germ-cell contains large irregular peripheral vacuoles, due to the solution of the yolk-content. A cell from the superjacent mesenchyma is shown at (*a*) for purpose of comparison between the nuclei of the germ cells, entodermal cells, and more differentiated mesenchymal cells. ×1,500.

FIG. 12.—Germ-cell from entoderm of dorsal region of hind-gut, of a 7-day embryo. The cytoplasm contains enormous peripheral vacuoles, where the yolk-globules have been dissolved. ×1,500.

FIG. 13.—Germ-cell in the entoderm from about mid-line of area pellucida in a 3-day embryo. The cell is in the act of migrating into the superjacent visceral layer of the lateral plate of mesoderm. ×1,500. The arrow points towards the embryonic axis.

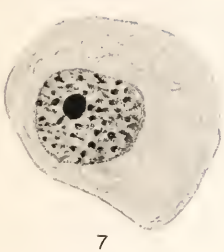
FIG. 14.—Germ-cell of a 3-day embryo (fig. 1, plate III), from area pellucida near its lateral border. The cytoplasm contains two large vacuoles where some of the yolk material has been dissolved. This cell has a diameter of 16.5 microns. The germ-cells vary from 13 to 22 microns in diameter.

FIG. 15.—Germ-cell from area opaca, near the margin of the area pellucida, of a 3-day embryo. For comparison a nucleus from the entodermal syncytium is added at *a*. Note the close similarity of structure. The cytoplasm of the germ-cell contains large spherical yolk-globules, which at this stage of development were not dissolved in the alcohols after Helly fixation (iron-hematoxylin stain). Only at later stages, when the yolk has suffered further changes in the process of elaboration for nutritive purposes, does it become susceptible to the solvent action of the alcohols. ×1,500.



GERM CELLS IN CARETTA

A Hoen & Co Lith.



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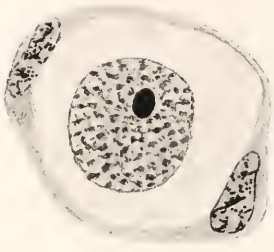

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a

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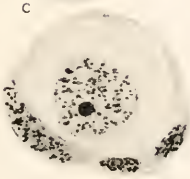
c



b

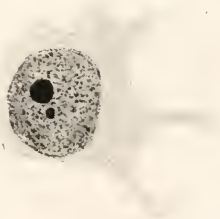
d

Visceral

a

Mesoderm

11



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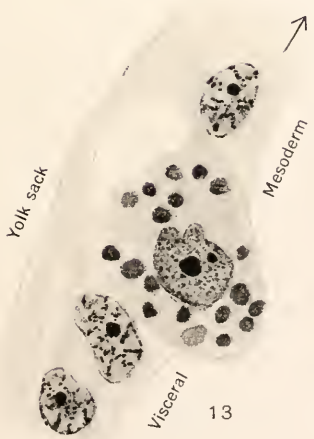
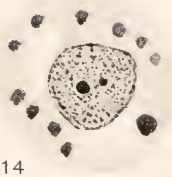


Yolk sack

Mesoderm

Visceral

13



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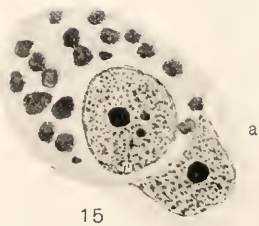


a

15

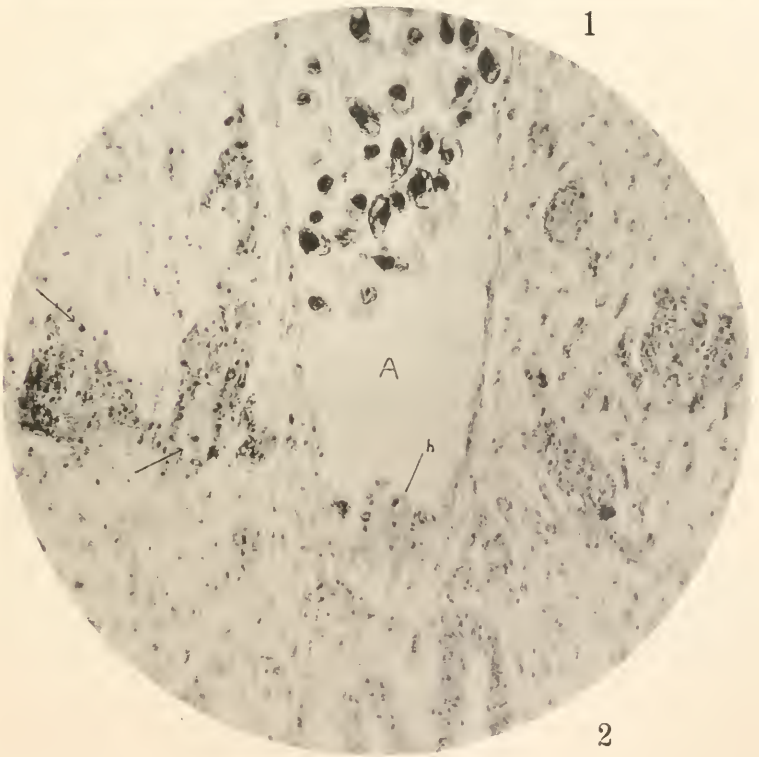
A Hoen & Co Lith.

GERM CELLS IN CARETTA

1. Transverse section of a 3-day embryo at level of tenth somite. The arrow points to the cord of primordial germ-cells, extending from the sixth somite to caudal extremity of primitive streak, at the lateral border of the area pellucida. Helly fixation; iron-hematoxylin stain. Photo. x 40

2. Transverse section of a 5-day embryo at level near cephalic extremity of the entodermal cords of primordial germ-cells. The two bilateral cords are more or less interrupted or segmented. This section passes through an intersegmental region. Certain germ-cells are also scattered more medially among the entodermal cells of the area pellucida. Arrows point to germ-cells. Helly fixation; iron-hematoxylin stain. Photo. x 66

1



2

1. Transverse section of same 5-day embryo at a level 300 microns forward of section fig. 2, plate 3, through the cord of segregated germ-cells (A) at left. The cord has made a linear connection with the overlying visceral mesoderm and the germ-cells are migrating into it and moving medially. Photo. x 100.
2. Section through abdominal aorta and adjacent masses of sympathetic neuroblasts, 150 microns behind section fig. 3, plate 6. The arrows point to the primordial germ-cells among the neuroblasts. (A) aorta filled with blood dorsally; (*h*), hemoblasts separating from endothelium ventrally. Photo. x 400. (Helly fixation; iron-hematoxylin stain.)

1. Transverse section of 9-day embryo and attached yolk-sac near level of cephalic extremity of mesonephros. The arrows point to different locations of the germ-cells (illustrated in figs. 1, 2, 3, and 4, Plate 1). Helly fixation, Giemsa stain. Photo. x 40. The extraneous material consists of broken yolk-globules.

2. Transverse section of same 9-day embryo at level of closed hind-gut, 250 microns caudad of preceding section, fig. 1. Arrows point to germ-cells. (*p. c. v.*), postcardinal vein; (*s. c. v.*), subcardinal vein; (*p. d.*), Wolffian duct. Photo. x 40. The dark irregular masses in this section consist of yolk-globules broken and scattered in sectioning.

1. Photomicrograph of a transverse section of 12-day embryo at level of anteriorly open hind-gut. The arrows point to different locations of primordial germ-cells (illustrated in figure 6, plate 1). (*p. d.*) Wolffian duct. x 47. The cœlomic cavity contains blood-cells and yolk-globules.
2. Transverse section at level of closed hind-gut of same 12-day embryo, 300 microns behind section, fig. 1. The arrows point to germ-cells. (*p. d.*), Wolffian duct; (*g.r.*), genital ridge. Photo. x 47. The cœlom contains blood and yolk globules.
3. Transverse section of same 12-day embryo, 550 microns caudad of fig. 2, at level of cloaca (*c*). (*s. g.*), sympathetic ganglion cells, among which a few primordial germ-cells have migrated. Arrows point to the locations of germ-cells. Photo. x 40.

XIV.

ATRESIA OF THE ESOPHAGUS IN THE EMBRYO OF THE LOGGERHEAD TURTLE (CARETTA CARETTA): A NORMAL DEVELOPMENTAL CONDITION.

By H. E. JORDAN,

Department of Anatomy, University of Virginia.

Four plates of nine figures.

ATRESIA OF THE ESOPHAGUS IN THE EMBRYO OF THE LOGGERHEAD TURTLE (CARETTA CARETTA): A NORMAL DEVELOPMENTAL CONDITION.

By H. E. Jordan.

INTRODUCTION.

In a series of embryos of the loggerhead turtle collected and used originally for a study of the history of the primordial germ-cells, it was noticed that the esophagus was solid for a greater or less extent, approximately from the point of origin of the respiratory anlage to its bifurcation into the bronchi, from the eleventh to the thirty-second day of incubation. At the latter stage the esophagus is still occluded at its oral end, though now fenestrated for a considerable extent caudally; and it seems probable that the esophageal atresia persists practically to near the end of the incubation period (8 weeks) at the level just behind the opening of the larynx.

The points of special significance in regard to this material are: (1) the relatively longer persistence of the occlusion than has yet been described for any other form; (2) the absence of contributory yolk in the stenosed area; (3) close relation to the point of origin of the respiratory anlage, which fact may disclose its possible functional significance.

Balfour was the first (1878) to describe a similar phenomenon in the esophagus of certain selachii. Kreuter (1903) confirmed these observations in the case of *Pristiurus* and *Torpedo*. Dean (1897) reports a solid esophagus in larvæ of *Amia calva*. An occluded esophagus is said to occur also in certain bony fishes—*e. g.*, herring, trout, salmon (Balfour, Oppel). In cyclostomes the esophagus remains patent throughout development (Kreuter). In certain amphibia (*Bufo*, *Rana*) the esophagus becomes occluded, in part through the medium of contributory yolk-globules (Meuron, 1886); and the same is true for certain reptiles (*Anguis fragilis*, Oppel, 1891; *Lacerta*, Meuron, 1886). According to Meuron, the esophagus of the chick embryo of the fifth day is occluded for a length of 115 microns, but regains partial patency again in the sixth day through the appearance of vacuoles. Lillie describes the esophagus of the chick embryo as completely occluded immediately behind the glottis from the eighth to the eleventh day, "owing to proliferation of the lining cells."

Kreuter (1905) was the first to describe an epithelial obliteration of the esophageal lumen in the human embryo, contrary to the teaching of Kollmann and other embryologists that no solid stage of the esopha-

gus occurred in mammals and in man. Kreuter describes also similar obliterated areas in the mid-gut and hind-gut of fetuses between the fourth and tenth weeks. In four human embryos, measuring from 8.4 to 16 mm., Lewis (1912) describes an esophagus whose lumen is pervious throughout; however, he describes vacuoles in the epithelial lining of these stages similar to those described by Kreuter as stages in the opening of the solid esophagus. But he regards an atresia of the esophagus in the human embryo as abnormal at all stages (p. 368).

It would seem that an embryonic normal atresia of the esophagus is a widespread phenomenon among vertebrates, and is essentially similar from elasmobranch fishes to man.

The phenomenon has not yet, as far as I am aware, been described for turtles, a circumstance which adds to the interest of this investigation; nor have its intimate spatial relationship to the respiratory anlage and its probable functional significance been hitherto pointed out.

This work is based chiefly on embryos of the loggerhead turtle, *Caretta* (*Thalassochelys*) *caretta*, but enough has been seen also in embryos of the snapping turtle (*Chelydra serpentina*) and of *Chrysemys marginata* to warrant the statement that in these forms a substantially identical process occurs.

In occasional pig embryos also, from 8 to 12 mm. length, I have noticed short areas of occlusion in the esophagus.

More or less extensive atresia occurs also in the stomach, duodenum, and other portions of the small intestine, large intestine, and rectum in certain of the above-named forms, especially in mammals. Of these regions the duodenum is most commonly occluded. The character of the occlusion and the manner of the reestablishment of patency are in general here also through the agency of originally discrete inter-epithelial vacuoles which gradually coalesce. In the turtle *Caretta* no other portions of the alimentary tract were at any stage similarly occluded.

MATERIAL.

The material consists of a series of 26 loggerhead-turtle embryos ranging from the second to the thirty-second day of incubation. It was collected in the summer of 1914 during a visit to the Laboratory of Marine Biology of the Carnegie Institution of Washington, located on Loggerhead Key, Florida. I am greatly indebted to Dr. Alfred G. Mayer for the excellent facilities afforded for securing and preserving a large number of specimens, approximately 200.

Two fixing fluids were employed almost exclusively, namely, the Zenker-formol modification of Helly and the strong chrom-aceto-osmic solution of Flemming. After the Helly fixation, different sets of sections were variously stained with the Giemsa solution, the iron-hematoxylin mixtures of Heidenhain, and the Delafield's hematoxylin and eosin combination.

DESCRIPTIVE.

In embryos up to the end of the eleventh day the lumen of the esophagus is still patent, but the epithelial lining is greatly thickened dorsally and the lumen is in consequence constricted (fig. 1).[1] Dorsally the lining epithelium consists of about 7 layers of spheroidal cells; ventrally it is only 3 to 4 layers thick, and the cells are cylindrical. Figure 1 is taken at the level of the oral end of the laryngo-tracheal evagination. Figure 2 is taken 150 microns caudally, at the point of bifurcation of the trachea into the bronchi. These levels are important landmarks, for they are approximately the limits of the later atresia. It should be noted also that the laryngo-tracheal anlage at this stage is patent throughout; at later stages the epithelial thickening may occasionally involve also its orifice and cause a temporary occlusion. Mitosis is very extensive among the entodermal lining-cells of this region, especially centrally.

By the end of the twelfth day the esophagus is practically solid (fig. 3) from the level of the origin of the tracheal anlage to the point of its bifurcation, about 250 microns. A minute central opening, approximately the diameter of a nucleus of the surrounding cells, may remain. By the thirteenth day this opening also has become closed. At this stage the trachea is open throughout. This stage is of prime importance, for it shows the manner of closure of the esophageal tube.

During succeeding days the extreme proximal end of the trachea (larynx) becomes solid, as well as the esophagus between the limits of the origin and bifurcation of the trachea. At the end of the sixteenth day the esophageal atresia extends through 1,680 microns, from a medial pharyngeal stenosis 300 microns behind the point of origin of the laryngo-tracheal tube to 720 microns behind the point of its bifurcation. Figure 4 is of the cephalic extremity of the atresia, and shows also the atretic condition of the trachea proximally. Figure 5 is of a section 300 microns caudal to figure 4. Vacuoles occur among the epithelial cells centrally; such are present to the point of transition into the stomach through 1,380 microns (fig. 6).

In the 25-day embryo the esophagus is closed through 2,650 microns. The initial point of closure is medially just behind the laryngeal opening. As compared with earlier stages (*e. g.*, the 16-day embryo), it seems that the cephalic extremity of earliest closure—*i. e.*, *over* or just behind the tracheal opening—has shifted slightly caudally so as to leave the trachea (larynx) freely pervious anteriorly to the closed esophagus. The medial (pharyngeal) closure rapidly spreads laterally, so that within 100 microns the laterally wide, dorso-ventrally much compressed esophagus is completely closed, except for the lateral extremities, which contain a small central aperture. These lateral

[1]The photomicrographs were made by Mr. William S. Dunn, Cornell University Medical School, New York.

apertures persist through about 600 microns, the extremities having meanwhile bent ventrally, giving the esophagus through about 600 microns a wide crescent-shape, about 200 microns behind which point vacuoles begin to appear within the epithelial lining. These vacuoles increase in number and in size, and coalesce towards the caudal extremity. Figure 7 is taken at the point of transition from the esophagus to the stomach.

In the 32-day embryo the originally solid esophagus is fenestrated—except for the extreme anterior extent, about 100 microns, just behind the opening of the larynx—because of numerous vacuoles, which enlarge and coalesce caudally. Figure 8 is taken from the point of transition to the stomach. This portion of the esophagus has the appearance of a meshwork with large irregular areolæ and delicate nucleated trabeculæ (fig. 9). It suggests somewhat notochordal tissue of an embryo of about the 16-day stage. The esophagus is closed medially in a manner similar to earlier stages, 110 microns behind the opening of the trachea (larynx into pharynx). It is completely closed except for minute lateral canals for 100 microns. The stenosed (vacuolated) area extends through 5.33 mm.

In 5 embryos of *Chelydra serpentina* examined the facts are as follows: In a 4.2-mm. embryo the esophagus is open throughout. In a 6-mm. embryo, which corresponds closely in development to the 13-day *Caretta* embryo, the esophagus is solid from a point just behind the origin of the tracheal anlage to its bifurcation, through 670 microns. In another 6 mm. embryo the solid esophagus opens again 240 microns short of the bifurcation of the trachea, extending through only 580 microns. In a 7-mm. embryo the esophagus is solid for 560 microns, from a point 150 microns behind the original tracheal evagination to a point slightly forward of the bifurcation. In another 7-mm. embryo the esophagus is closed from a point 240 microns behind the connection with the trachea to a point 80 microns forward of the bifurcation, through 800 microns. In both the 6 mm. and 7-mm. embryos numerous vacuoles occur in the epithelial lining. Practically identical conditions prevail in *Chrysemys marginata*. In the older embryos the primitive glottis also becomes obliterated through apposition and fusion of its lateral walls.

From the above it appears that after the eleventh day of incubation the esophagus of *Caretta* is solid throughout a greater or less extent until at least the thirty-second day stage. This secondary imperforate area extends from about the point of the connection between the foregut and the trachea to the point of bifurcation of the latter into the bronchi. The closure of the originally open esophagus is accomplished in part by cell-proliferation, chiefly in the dorsal wall of the esophagus, accompanied by a dorso-ventral compression of the cylindric tube into one of rectangular outline; and it progresses in a cephalo-caudal direc-

tion. The reestablishment of the lumen begins caudally and extends forwards, the last point of opening being thus at the laryngeal level. The opening is accomplished by a process of vacuolization of the epithelial lining. Judged by the time occupied in the effective vacuolization of the caudal portion of the solid esophagus, it seems reasonable to suppose that the laryngeal end remains closed until, or near, the time of hatching. A matter of cardinal importance concerns the fact that the first point to close in the primitive esophagus is medially at or just behind the level of the tracheal (laryngeal) connection, and the last point to open is just behind this same level. It would seem, on the basis of this observation, as if the object of the transient solidification of the esophagus was to close the opening of the trachea during the greater period of the embryonic development of the lung against the mid- and hind-gut as if to protect the lung against materials contained in the gut-tube, chiefly yolk-globules.

It remains to describe in detail the process of closure and the subsequent vacuolization of the atresia whereby the lumen becomes reestablished. For this purpose embryos of the eleventh, twelfth, sixteenth, and thirty-second days will be chiefly employed. In the light of these additional details the hypothesis above suggested, namely, that the solid esophagus in the turtle embryo has its meaning in relation to the developing lung, perhaps protecting it against the entrance of yolk and other material from the gut, will be discussed; and an attempt will be made to correlate conditions in the turtle with those described for other vertebrate forms, and to interpret the occasional esophageal stenosis in mammalian embryos in terms of a reptilian ancestry. Exactly the same series of stages, as indicated by the histologic structure, occurs in the esophagus of the 32-day embryo in a cephalo-caudal direction, as appears in the greater extent of the esophagus of embryos of successive stages of development as represented in the 11- to 25-day stages.

The esophagus of the 11-day embryo, as stated above, is open throughout. But the lumen has become considerably narrowed by reason of an increase in the number of layers of the lining-cells. The number of layers dorsally varies from seven to nine, cell boundaries being indistinct. The two outermost and the two innermost layers consist of cylindrical cells; the remaining layers comprise chiefly polyhedral and spheroidal cells. Division, both mitotic and apparently amitotic, is most active among the cells of these intermediate layers. The number of cell-layers ventrally and lining the laryngotracheal groove, as indicated by the number of layers of nuclei, is four; but this portion of the epithelium is apparently for the most part a pseudo-stratified columnar type. Mitotic figures are most numerous among the central layers of nuclei—that is, next the lumen.

Where the wide pharynx passes into the oral end of the esophagus the lumen becomes triangular in cross-section, the base directed dorsally. The esophagus at this level is lined by 5 to 6 layers of cells throughout. The peripheral and central cells are cylindrical in shape, those of the intermediate layers have a polyhedral form, and many of the latter are in mitosis. At a slightly lower level the dorsal portion of the lining epithelium thickens, while in the ventral portion, *i. e.*, along the tracheal evagination, the epithelium becomes somewhat thinned. The shape of the lumen at this level, including here in continuity a portion belonging to the esophagus proper and a portion belonging to the laryngo-tracheal groove, is oval in transection, flattened from side to side.

At the level of bifurcation of the trachea the lumen of the esophagus has a narrow, spindle-shaped outline, compressed from side to side. This lumen is lined by a pseudo-stratified columnar epithelium, containing 4 rows of nuclei dorsally and 2 to 3 ventrally. The respiratory anlage, of rectangular outline, flattened dorso-ventrally, is lined by a similar type of epithelium with about 3 rows of nuclei. Mitosis is active among the cells immediately surrounding the lumen. Terminal bars are conspicuous, especially in the tracheal anlage.

The ventral laryngo-tracheal groove becomes separated from the primitive esophagus, and is consequently converted into a tube, by a process involving the approximation and ultimate fusion of bilateral mesenchymal plates, which process extends in a caudo-oral direction. In the area of the advancing residual connection between the separating tracheal and esophageal tubes, *i. e.*, at the level of constriction preceding the separation, the cells are evidently under considerable pressure. The nuclei are in general much smaller, of very variable size, and greatly crowded. They are spheroidal in outline and many show signs of degeneration. Many of these nuclei are vesicular and practically achromatic, except for a relatively large and very chromatic nucleolus. None of these cells are in mitosis, but many of the nuclei are at some phase of amitotic division. In the surrounding mesenchyme, however, especially in the plates which invaginate the esophageo-tracheal tube in the process of separation, mitosis is very active. Along the line of separation, the nuclei in the ventro-medial epithelium of the esophagus and in the dorso-medial epithelium of the trachea are much smaller, more nearly spherical, and more crowded than elsewhere in these tubes. A few smaller spheroidal yolk-globules are present in the lining-cells of both the esophagus and trachea at these stages, but they are relatively more numerous in the esophageal epithelium.

In the 12-day embryo the esophagus practically lacks a lumen (fig. 3). At the level just behind the point of connection between the primitive esophagus and the trachea (definitive pharynx and larynx) the narrow circular lumen of the trachea is lined with 3 to 4

layers of cells, some of which, especially centrally, are in mitosis. The esophagus, just cephalad of this point (*i. e.*, in the region of the glottis) has a T-shaped lumen, lined dorsally with about 9 layers of cells, of cylindric form peripherally and centrally and of polyhedral form in the intermediate layers. Viewed as a whole—*i. e.*, the lumen and its cells—the esophagus is still more distinctly T-shaped, the cross-bar in the esophagus proper being about equal to or even greater in length than the stem or the laryngeal anlage.

At the level here illustrated, figure 3, the minute central opening of the esophagus is surrounded by only 3 to 4 layers of cells, as indicated by the rows of nuclei. By the thirteenth day even this capillary lumen is obliterated and the esophagus is completely closed for about 0.5 mm. This closure is apparently effected by a process of lateral traction (or possibly dorso-ventral compression) upon the originally cylindric tube, causing thus a change in shape of the lumen from an oval or a circular to a horizontal cleft form. This mechanical factor is assisted by the larger number of cell-layers dorsally, the result of greater proliferative activity, and accomplishes finally a close apposition and ultimate fusion of the dorsal and ventral walls. One small lateral vacuole was seen in the epithelial lining of the stenosed esophagus at the 12-day stage; at the 13-day stage three lateral vacuoles had appeared.

The central area of the closed esophagus of this stage consists of irregular stellate cells, resembling a compact mesenchymal reticulum. These cells come chiefly from the dorsal thicker wall, and represent modification products of originally cylindric cells.

At the 16-day stage of development the esophagus is closed for approximately 1.5 mm. At the most anterior point of occlusion the pharynx is wide and somewhat crescentic, with the concavity directed ventrally. The occluded area at this level occupies only the smaller medial portion of the wide pharynx at the point where it passes into the esophagus. The larynx at this level has an oval, slit-like lumen, compressed laterally and lined with a pseudo-stratified columnar epithelium containing 2 to 4 rows of oval nuclei, many of which centrally are in mitosis. The pharynx is lined with columnar epithelium (apparently pseudo-stratified) with 3 rows of nuclei, oval in shape peripherally and centrally. At the most cephalic point of the closure the pharynx remains widely open laterally. Only a few mitotic figures appear among these cells. In the median fused area the central cells are of irregular shape, with spheroidal nuclei, and form a mesenchyme-like syncytium. At this stage the glottis is closed for a short distance, due probably to the pressure exerted upon this portion of the tube by the surrounding mesenchyme in the process of final cephalic division of this portion of the fore-gut into esophagus and trachea. The fusion of the lateral walls of the larnyx and the conse-

quent obliteration of the lumen are probably due in part also to the arytenoid swellings just appearing in this region.

Vacuoles first appear, either singly or in groups, centrally and laterally. These are the areas where the fusion-reticulum is originally less dense, because here the original lumen persisted longest. As the vacuoles form, the nuclei of the syncytium become disposed in the manner of an epithelium around these spaces, as if rearranged under pressure from within the vacuoles. The original smaller vacuoles grow in size and coalesce with adjacent vacuoles. Only very rarely is a mitotic figure seen among the central cells. Mitosis is slight at this stage, also among the peripheral cells. An extremely small, practically negligible, amount of cell-degeneration occurs among the central cells. The vacuoles tend to become confluent in a lateral direction; they are apparently empty and contain no coagulum in stained sections. Their spheroidal shape and the manner of the arrangement of the enveloping cells leave no room for doubt, however, that they were formed, at least in large part, under the influence of a fluid pressure.

In the 25-day embryo the vacuoles are larger and more numerous, and they increase in number and size caudally, where a fenestrated condition of the atretic lumen has become established.

Coincident with this fenestration, the enveloping mesenchyme (tela submucosa) of the esophagus has become looser and more vascular, thus permitting more readily an expansion of the tube under the pressure produced internally during the formation of the vacuoles. This expansion is assisted also no doubt by the growth, as indicated by extensive mitotic activity, of the peripheral portion of the wall, and possibly in part also by external traction exerted by the growing mesenchyme.

At the 32-day stage the lumen of the esophagus is in the fenestrated condition throughout its greater extent, only the most cephalic portion remaining practically solid. The lumen appears to be spanned across by delicate nucleated septa with lateral anastomoses. Where the septa join the main wall of the lumen they spread into triangular multinucleated bases. There is no sign of degeneration among the nuclei of these septa—in fact, a number of the nuclei may be in process of either mitotic or amitotic division. The trabeculæ are subsequently simply drawn into the peripheral portions of the wall and gradually incorporated among the entodermal cells of the lining epithelium.

DISCUSSION.

A complete discussion of the phenomenon of esophageal atresia here described must take into special consideration the following facts:

(1) The inception of the atresia at the 12-day stage is coincident with the early stages of the formation of the respiratory anlages.

(2) The initial point of stenosis is spatially very closely related to the orifice of the developing laryngo-tracheal tube.

(3) The transient solidification of the esophagus progresses in a caudal direction, while the reestablishment of patency progresses in the opposite direction.

(4) The early stenosis of the esophagus, from the eleventh to the twelfth day, results largely from an alteration in the shape of the esophagus, from a tube approximately circular in cross-section to one flattened dorso-ventrally into a rectangular structure with at first a slit-like lumen and later a minute central circular aperture; the stenosis becomes an atresia at the thirteenth day through a fusion of the dorsal and ventral walls, the cells involved becoming converted into a mesenchyme-like syncytium; this process is assisted by the increase in the number of cell-layers dorsally, where mitotic activity is extensive; the factors involved in the change of shape of the esophagus are largely mechanical, incidental to inherent and extraneous tissue growth, the chief element in forcing the growth of the esophageal tube into an adaptive rectangular form being most probably the denser bilateral mesenchymal plates by whose medial approximation and fusion the original esophageo-respiratory anlage becomes converted into the esophageal and tracheal tubes.

(5) The esophageal atresia involves also a transient stenosis and atresia of the oral end (glottis) of the respiratory anlage.

(6) The definitive perforate esophagus becomes established by a process involving the transformation of certain "intercellular" spaces in the central syncytium into spherical vacuoles, and the enlargement and confluence of these to form larger irregular areolæ; at this stage (32-day) the relatively wide esophageal lumen is spanned by delicate nucleated septa; these septa are ultimately drawn into the expanding peripheral wall and here incorporated with practically no tissue degeneration; the earlier spherical shape of the vacuoles and the disposition of the nuclei around these spaces indicate their formation under the influence of an internal fluid pressure, but no coagulum appears in fixed and stained tissues; the processes of later vacuolization and incorporation of the intra-luminal septa by the peripheral wall are probably largely the results of the growth expansion of the esophageal tube resulting from active proliferation of the lining-cells and assisted by the now very loose and vascular enveloping mesenchyme, the primitive submucosa of the esophagus.

(7) The lumen of the esophagus is still occluded just behind the laryngeal orifice at the 32-day stage and no doubt remains stenosed until near the close of the incubation period (56 days).

(8) All forms in which an extensive atresia of the esophagus has been reported (elasmobranchii, bony fishes, amphibians, certain reptiles, chick) have the gut open to a huge yolk-sac during the greater portion of the incubation period.

The above-enumerated facts would seem to show conclusively that in the *Caretta* embryo the temporary atresia of the esophagus is a normal phase of the developmental process. This is probably true also of all forms with large yolk-sacs. The occasional esophageal constrictions, stenoses, and atresias in the pig embryo, other mammalian embryos, and in man (a normal phenomenon according to Kreuter, abnormal according to Lewis) probably have their explanation in terms of their reptilian ancestry. These conditions in mammals are apparently very variable and any discussion as to their normality or abnormality in these embryos seems of small value. Congenital stenoses or atresias of the esophagus in humans, as Kreuter has already suggested, represent most likely a persistence or exaggeration of a normal phase of embryonic development.

A suggestion of the teleological significance of this temporary atresia of the esophagus in *Caretta* may be obtained by seeking to disclose what is actually accomplished by the phenomenon. Obviously, it closes the respiratory anlage against the more solid contents of the gut during the greater portion of the incubation period. That the relation of the obliteration of the lumen of the esophagus is close to the development of the respiratory anlage is strongly indicated by the following facts:

(1) Its first appearance about the time the laryngo-tracheal groove begins to be separated from the esophagus and at or just behind the later orifice of the larynx.

(2) The extension of the atresia even into the glottis.

(3) The persistence of the atresia orally until nearly the end of the incubation period, when the lung is already greatly developed, and the yolk is almost entirely digested.

It is difficult to avoid the inference that the temporary solidification of the esophagus is a device to protect the developing lung against the invasion from the gut of yolk-globules. The respiratory anlage, being a derivative of the primitive esophagus, is originally lined by an epithelium which retains in part the ability to digest yolk material; but soon after, as it becomes more and more differentiated into the respiratory type of entodermal epithelium, it must more and more lose the capacity for digesting crude yolk material. It seems reasonable to suppose that large quantities of yolk-globules within the pulmonary anlages would seriously interfere with their normal development. That this

is actually the correct interpretation of the significance of the solid esophagus in forms with telolecithal, especially meroblastic, eggs could be definitely established only on the basis of experimental evidence; but lacking such, the available facts speak strongly in favor of such a view. In forms with meiolecithal eggs such a protective mechanism against gut-yolk is superfluous; and it is apparently lacking except in slight and variable degree. A congenital atretic esophagus in mammals could be explained in terms of the persistence or exaggeration of a normal or anomalous embryonic condition, the ontogenetic expression of a phylogenetic experience.

As already described, the obliteration of the esophageal lumen (contrary to Kreuter) is only partly the result of excessive cell proliferation, this being limited to the dorsal wall. The chief factor is a change in form of the cylindric tube, effected by the growth of the esophagus operating against the confining plates of denser mesenchyme, which brings about the separation of the ventral portion of the original esophageo-tracheal anlage to form the laryngo-tracheal tube. The thickened dorsal and the ventral walls of the esophagus are thus brought into apposition and finally fuse, thus obliterating the lumen by a central syncytium.

Contrary to what one might expect *a priori*, the lumen of the esophagus is reestablished without any tissue degeneration. This statement is in agreement with the observations both of Kreuter and of Lewis for human embryos. Lewis believes that the constricted lumen of the esophagus in the human embryo becomes enlarged by a process involving a shifting of mitotic activity from the central layer of cells to the peripheral layers, with vacuoles forming as incidental intermediate phenomena. In the *Caretta* embryo there appears no striking evidence to indicate that such a process enters as a large factor. It is true that in the stages preceding the atresia mitotic figures are most numerous centrally, and again that during the reopening of the lumen mitosis is extensive among the peripheral cells; but mitosis is by no means limited to these regions, and the relatively slight excess in one region or the other at the different periods seems to me quite insufficient to account for the early closure and the later vacuolization of the esophagus in the *Caretta* embryo.

The chief factor in the closing process is the change in shape of the lumen, which brings the dorsal and ventral walls into contact and results in a fusion. And the main factor in producing the early vacuolization would seem to be the collection of fluid in the "intercellular" spaces of the central syncytial mass. The earlier vacuoles are uniformly spherical and the cells are arranged about them in the manner of an epithelium, such as would result if drops of fluid grew in size among compacted cells. The irregular condition of the later vacuolization indicates, however, that another factor now becomes

chiefly active—namely, one producing traction upon the walls of the vacuoles from without—thus changing them into more or less delicate and anastomosing septa. This factor is no doubt inherent in the growth of the circumference of the esophagus, the mechanism being enabled to exert a maximum expansive effect by reason of the now very loose, wide, and vascular enveloping mesenchyme, the primitive tela submucosa. In agreement with the observations of Kreuter and of Lewis in the case of human embryos, the vacuolization of the esophageal epithelium involves no tissue degeneration in *Caretta*. Occasional nuclei of the septa are in mitosis, and the whole meshwork, including even the most delicate trabeculæ, is ultimately drawn into the peripheral epithelial wall and thus incorporated among the entodermal cells.

SUMMARY.

1. A series of 26 loggerhead-turtle embryos, ranging from the second to the thirty-second day of incubation, were available for this study. Atresia of the esophagus is initiated during the twelfth day of incubation. The observations are made chiefly on the 11, 12, 13, 16, 20, 25, and 32 day stages, and the conclusions are based chiefly on this selected material.

2. During the tenth and eleventh days of incubation the epithelial lining of the oral end of the esophagus (esophageo-respiratory anlage) thickens greatly dorsally, the result of extensive cell proliferation in this region. During the twelfth day the cylindric tube of the esophagus becomes compressed dorso-ventrally, thus bringing the dorsal and ventral epithelial walls in close apposition. Only the minutest central lumen persists in the oral end of the esophagus for a distance of about 0.25 mm. During the thirteenth day the oral end of the esophagus is rectangular in cross-section and completely solid for a distance of about 0.5 mm. The apposed central cells have fused and have formed a plug of tissue, essentially a mesenchyme-like syncytium.

3. The initial point of atresia is *over*, or *just behind*, the orifice of the separating laryngo-tracheal anlage; and its inception is coincident with the earliest stage in the division of the original esophageo-respiratory anlage into an esophageal and a laryngo-tracheal tube. By the sixteenth day the atresia has extended into the orifice of the larynx, due in part perhaps to pressure exerted by the lateral arytenoid swellings.

4. The chief factor in the temporary closure of the originally open esophagus is the change in shape of the esophagus from a tube approximately circular in cross-section to a structure of wide rectangular form with at first a slit-like lumen and finally a minute central aperture. The cause of the change in shape, upon which the obliteration of the

lumen largely depends, is the combination of growth within the esophagus in opposition to the denser lateral mesenchymal plates, by the invasion and medial fusion of which the laryngo-tracheal groove becomes converted into a tube and incidentally separated from the esophagus distally. This process is assisted, as concerns the obliteration of the esophageal lumen, by the active cell proliferation in the dorsal wall of the esophagus.

5. In the 16-day embryo the atresia of the esophagus extends through about 1,500 microns. Beyond the oral end vacuoles begin to form in the lining epithelium. These represent dilated "intercellular" spaces chiefly within the central syncytial plug of tissue. They increase in number, and enlarge caudally, where they become confluent. During succeeding stages this process of vacuolization continues, until at the 32-day stage only the extreme oral end of the esophagus remains closed.

6. Both the closure and the reestablishment of the lumen of the embryonic esophagus involve mechanical as well as growth processes, but are normal for a certain stage of the embryonic development. The closure is not largely dependent upon intrinsic cell-division, and the fenestration process involves no tissue degeneration or resorption. The level of initial closure and the level of final perforation are approximately the same—namely, the laryngeal level of the esophagus.

7. In the process of vacuolization upon which the opening of the temporarily stenosed esophagus depends, the larger spherical vacuoles are drawn into irregular spaces as if through traction exerted from without. This traction no doubt inheres in the growing and expanding periphery of the esophagus. The esophagus now has a fenestrated appearance in section; its lumen is spanned by more or less delicate nucleated septa which may anastomose, giving to the whole the appearance of a wide-meshed syncytium. Ultimately the trabeculæ are drawn into the lining epithelium and their nuclei incorporated among the entodermal cells of the mucous lining.

8. The temporary atresia of the esophagus in the *Caretta* embryo would appear to be a device for the protection of the lung during its development against yolk material from the gut, which material could not be digested, but would interfere with normal development of the lung.

9. This hypothesis can comprehend and correlate conditions in embryos of forms with meroblastic, holoblastic telolecithal, and alecithal eggs. Where yolk is very abundant, as in the meroblastic eggs of fishes, reptiles, and birds, the atresia is relatively extensive and of longer duration; in amphibia the closure is largely of the nature of a stenosis in which yolk-globules are involved, probably in process of digestion, while their forward progress is delayed by reason of the con-

stricted lumen. In most mammals and in man such mechanism is functionally superfluous, and consequently absent except in slight and variable degree. As such it may persist or become accentuated, and produce congenital atresia or stenosis of the esophagus. The relatively frequent embryonic and congenital occlusions of the duodenum just caudal to the pancreatic anlages may perhaps likewise find their explanation in terms of a phylogenetic protective device against yolk invasion.

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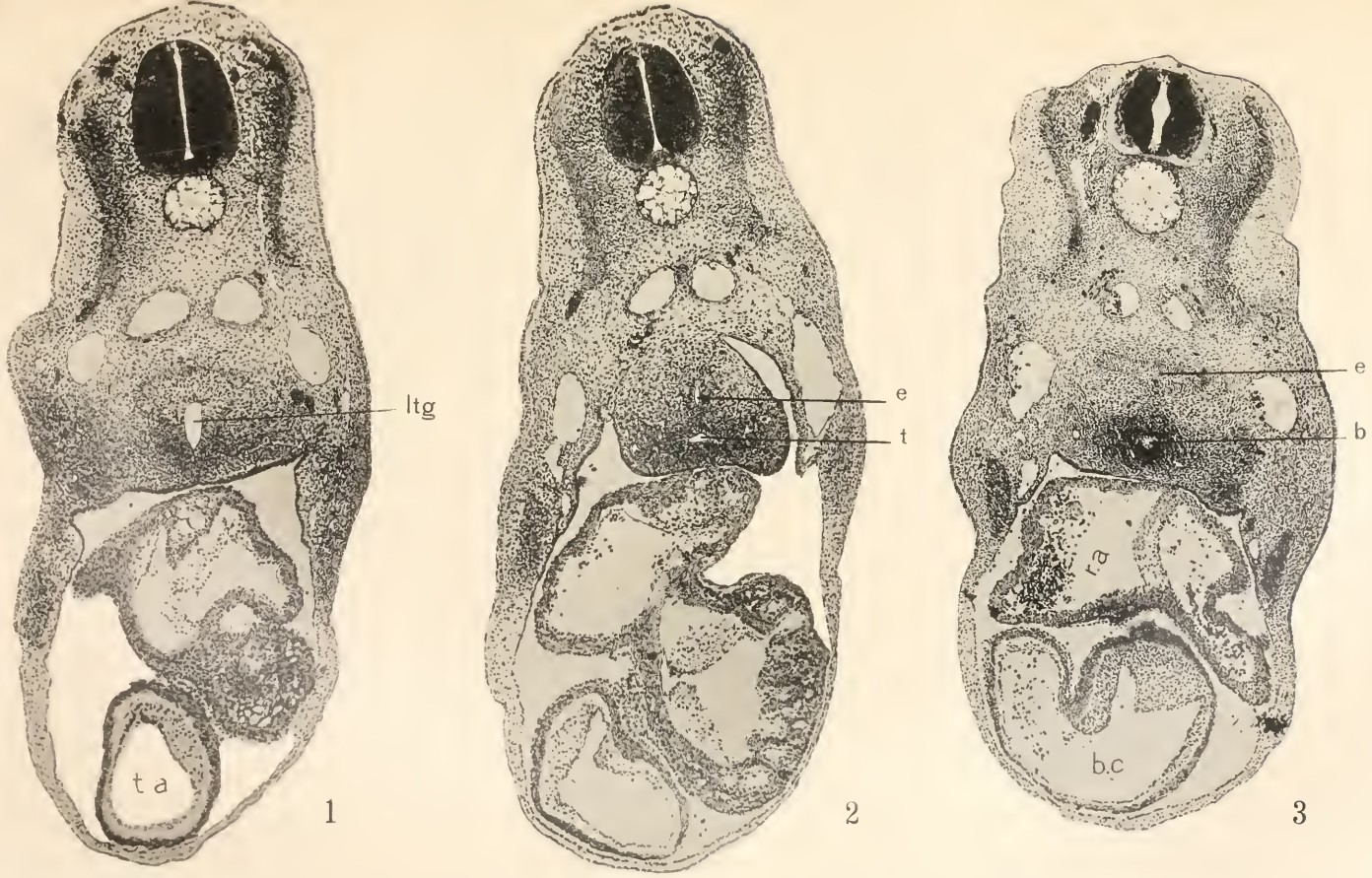
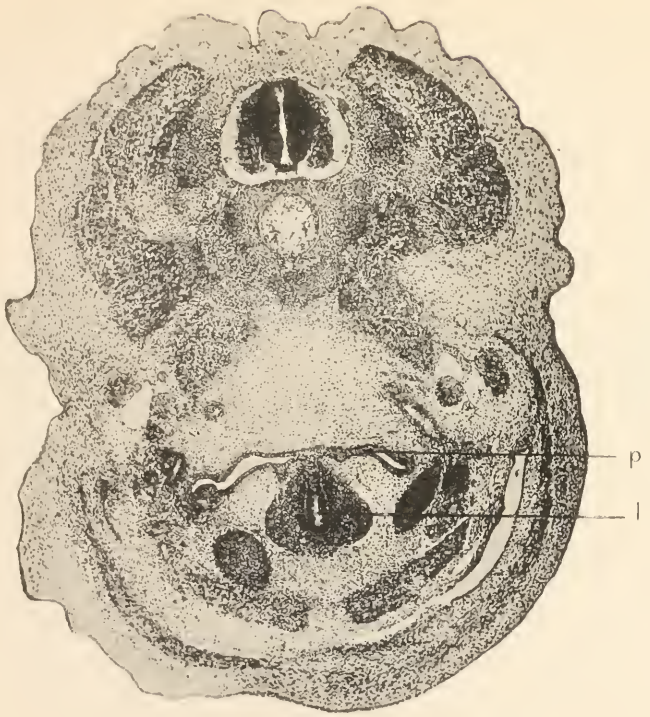


1. Photomicrograph of transverse section of 11-day loggerhead-turtle embryo at level of cephalic extremity of laryngo-tracheal groove (*ltg*). Note the epithelial thickening in dorsal wall of esophagus; (*t. a.*), truncus arteriosus. Just dorsal to esophagus are the dorsal aortae; laterally lie the anterior cardinal (jugular) veins. x 38
2. Transverse section of same embryo, 150 microns caudad of level shown in fig. 1, at point where trachea (*t*), still connected with the esophagus by an epithelial plate, bifurcates into two primary bronchi. (*e*), esophagus, with patent lumen, and oval outline at this level. The line (*e*) passes across left duct of Cuvier. Photo. x 38.
3. Transverse section of 12-day embryo at approximately same level as that shown in fig. 2. The esophagus (*e*) has suffered compression dorsoventrally and has become rectangular in outline (similar to its more cephalic condition in 11-day embryo): its lumen has become obliterated through fusion of its dorsal and ventral epithelial walls. (*b*) trachea at the point of bifurcation into the bronchi; (*r.a.*) right atrium; (*b.c.*) bulbus cordis. Photo. x 38.



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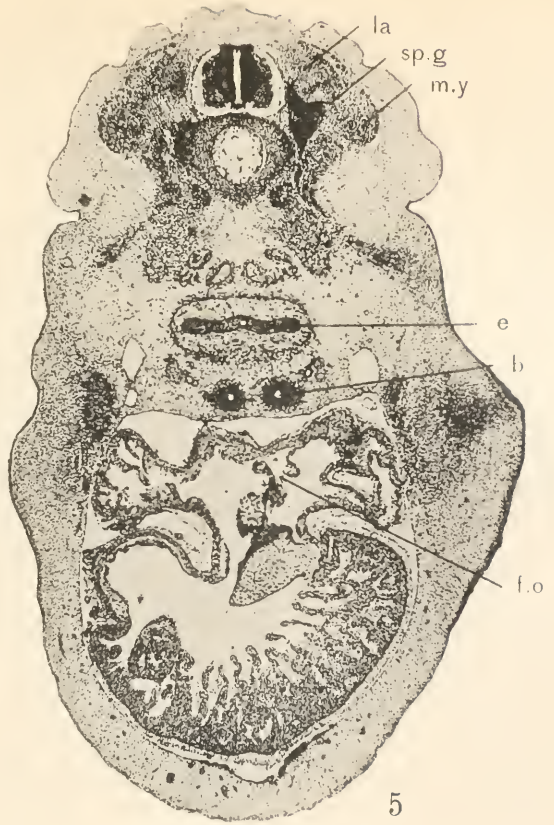

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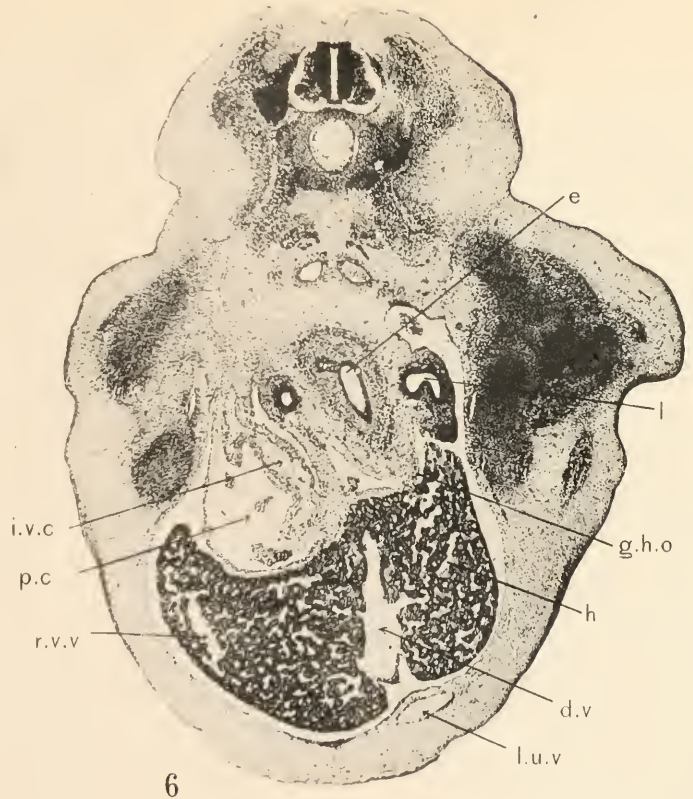
4. Transverse section of 16-day embryo at level of transition from pharynx (*p*) to esophagus, showing most anterior point of medial fusion of walls of esophagus. Note that the pharyngo-esophageal atresia has extended into the proximal portion of the laryngeal lumen (*l*) or glottis. The mesenchymal swellings on either side of glottis are the arytenoid anlages. The initial point of closure of the esophagus is at level of laryngeal opening. This is also the level where patency is last reestablished towards end of the incubation period. Photo. x 38.

5 Transverse section of the same embryo, 300 microns caudad of section fig. 4. The esophagus (*e*) has here a rectangular form in section greatly flattened dorsoventrally. The periepithelial mesenchyme (anlage of tela submucosa) has become much looser and more vascular and a more peripheral muscle-layer is beginning to form. The esophagus is solid except for a few small disconnected vacuoles, among the entodermal lining-cells. These vacuoles are dilated intercellular spaces. (*b*), primary bronchus; (*f. o.*), foramen ovale. (*l a.*), lamina of avertebra (cartilaginous stage); (*m.y.*), myotome; (*sp. g.*), spinal ganglion. Photo x 27.



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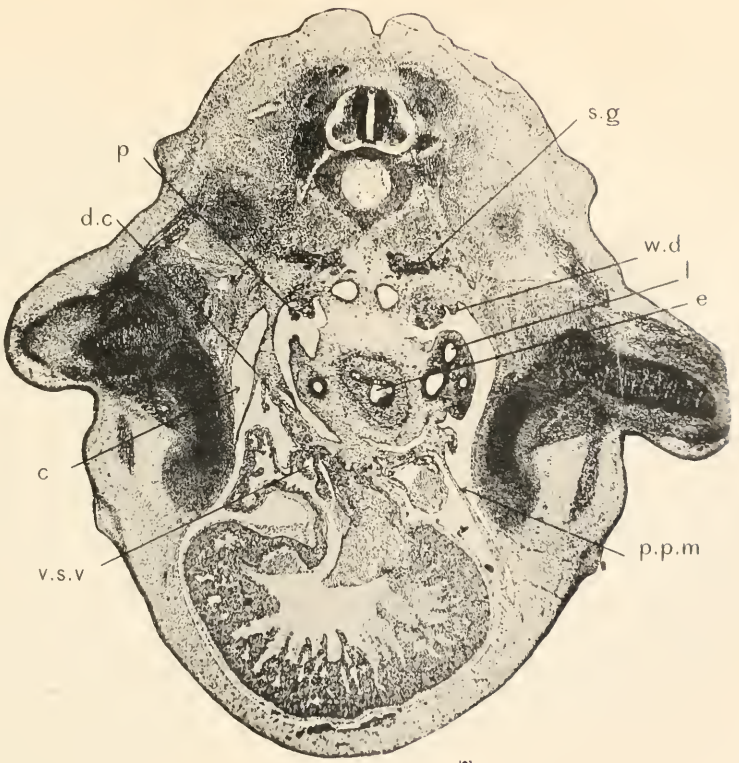

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6 Transverse section of same 16-day embryo at level of transition of esophagus to stomach (*e*), 1200 microns caudad of section fig. 5. Vacuoles may be seen in the atretic esophagus caudally and in lining of the cardiac extremity of the stomach. (*l*), left lung; (*g. h. o.*), gastro-hepatic omentum, (*h*), liver; (*d. v.*), ductus venosus; (*l. u. v.*), left umbilical vein, (*i. v. c.*), inferior vena cava, (*p. c.*), pericardial cœlom; (*r. v. v.*), right vitelline (omphalomesenteric; hepatic) vein. Photo. x 27.

7. Transverse section of 25-day embryo at level of transition from esophagus to cardiac end of stomach (*e*). The lumen of the esophagus is beginning to be reestablished caudally by a process of vacuolization and a fusion of the vacuoles, without sign of tissue degeneration. The loose and vascular character of the enveloping mesenchyme permits the dilatation of the esophagus necessitated by this process. (*s.g.*), sympathetic ganglion; (*w. d.*), cephalic extremity of fold lodging the Wolffian duct; (*l.*), left lung; (*p. p. m.*), pleuro-pericardial membrane; (*p.*), pronephros; (*d. c.*), right duct of Cuvier; (*c*), cœlom (dorsal parietal recess); (*v. s. v.*), venous valves guarding the opening of right cornu of sinus venosus into the right atrium. Photo. x 20.

8. Sinistro-dorsal portion of transverse section of 32-day embryo at level of transition from esophagus (*e*) to the stomach. The apparent obliquity of the section is due to the sinistral rotation of the cardiac end of stomach (*c. s*). The approximately transverse position of stomach at these stages accounts for its being cut longitudinally in a transverse section of the embryo. Note the extensively fenestrated condition of the caudal portion of the esophagus (*e*, within the rectangle) at this stage. (*r*), rib; (*c*), pleural cœlom; (*l*), left lung; (*a*), right dorsal aorta; (*b*), right primary bronchus. Photo. x 17.

9. More highly magnified photomicrograph (x 300), of portion of esophagus included within rectangle in preceding figure. (*x*), earlier irregular and several-layered condition of lining epithelium of esophagus during period of fenestration of solid esophagus in the process of reestablishment of patency of its lumen; (*y*), a delicate nucleated septum, in process of reincorporation within the later regular single-layered lining of the open esophagus; (*z*), portion where lumen is lined with the later (transient) single-layered epithelium of prismatic cells.

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